PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date f mailing (day/month/year) 17 February 2000 (17.02.00)	
International application No. PCT/US99/10821	**Applicant's or agent's file reference** 7024381Pur92
International filing date (day/month/year) 18 May 1999 (18.05.99)	**Priority date** (day/month/year) 18 May 1998 (18.05.98)
Applicant PAK, William, L. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

15 December 1999 (15.12.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau f WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer C. Cupello
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

3117287

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/435, C12N 1/00, 5/10, 15/12, 15/63, C12P 21/00

A1

(11) International Publication Number: **WO 99/60022**

(43) International Publication Date: 25 November 1999 (25.11.99)

(21) International Application Number: PCT/US99/10821

(22) International Filing Date: 18 May 1999 (18.05.99)

(30) Priority Data:

60/087,368	18 May 1998 (18.05.98)	US
60/098,072	27 August 1998 (27.08.98)	US

(71) Applicant *(for all designated States except US)*: PURDUE RESEARCH FOUNDATION [US/US]; Office of Technology Transfer, 1063 Hovde Hall, West Lafayette, IN 47907 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: PAK, William, L. [US/US]; 1025 Windwood Lane, West Lafayette, IN 47906 (US). LI, Chenjian [CN/US]; 221 Carrollwood Drive, Tarrytown, NY 10591 (US). GENG, Chaoxian [CN/US]; 228 Arnold Drive, West Lafayette, IN 47906 (US).

(74) Agents: SCHWARTZ, Jason, J. et al.; Woodard, Emhardt, Naughton, Moriarty & McNett, Bank One Center/Tower, Suite 3700, 111 Monument Circle, Indianapolis, IN 46204 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: CALCIUM CHANNEL REGULATORS

(57) Abstract

Purified InaF proteins that function in ulating calcium ion entry into cells are provided. Nucleotide sequences encoding functional InaF proteins are also provided. The invention also provides recombinant vectors including the nucleotide sequence encoding InaF, host cells that include the recombinant vectors described herein and methods of expressing InaF proteins by culturing such host cells.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10821

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 14/435; C12N 1/00, 5/10, 15/12, 15/63; C12P 21/00
US CL : 435/69.1, 320.1, 252.3, 325, 410; 530/350; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 320.1, 252.3, 325, 410; 530/350; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	Database EMBL-est58-GENBANK-est111, Accession Number AI403010, HARVEY et al. 'BDGP/HHMI Drosophila EST Project', 08 February 1999, see entire document.	1-43
X, P	CHAOXIAN et al. A novel protein required for store-operated calcium entry. Society for Neuroscience Abstracts. 1998, Vol. 24, page 2030, abstract 812.1, entire abstract.	1-43
A	MONTELL, C. New light on TRP and TRPL. Molecular Pharmacology. 1997, Vol. 52, pages 755-763, entire document.	1-43
A, P	MONTELL, C. TRP trapped in fly signalling web. Current Opinion in Neurobiology. June 1998, Vol. 8, No. 3, pages 389-397, entire document.	1-43

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
04 AUGUST 1999	09 SEP 1999

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	GABRIELE ELISABETH BUGAISKY
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10821

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

pir, genbank, embl, Swiss-prot, A-geneseq, N-geneseq; Dialog files 5, 155, 35 (Biosis, Medline, Diss. Abs.); APS, CAS-STN files registry, caplus
search terms: Calcium, channel, eye#, drosophila, melanogaster, mqqqrqqllqrqh/sqsp, inaf, ina##, 10c##, 10d##, 10e##, X(3a)chromosome, store(w)operat?, capacit?, trp

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET
Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference: 7024381Pur92

Applicant: PURDUE RESEARCH FOUNDATION, etal

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . 240 [T]

2. SEARCH FEE . . . . . 700 [S]

International search to be carried out by US

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains 81 sheets.

first 30 sheets . . . . . 455 [b1]

51 (remaining sheets) x 10 (additional amount) = 510 [b2]

Add amounts entered at b1 and b2 and enter total at B . . . . 965 [B]

Designation Fees

The international application contains 79 designations.

10 (number of designation fees payable *(maximum 10)*) x 105 (amount of designation fee) = max. 1050 [D]

Add amounts entered at B and D and enter total at I . . . . . 2015 [I]

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* . . . . . 30 [P]

5. TOTAL FEES PAYABLE . . . . . 2985 TOTAL

Add amounts entered at T, S, I and P, and enter total in the TOTAL box

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

- ☒ authorization to charge deposit account (see below)
- ☒ cheque
- ☐ postal money order
- ☐ bank draft
- ☐ cash
- ☐ revenue stamps
- ☐ coupons
- ☐ other *(specify):*

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ US

- ☐ is hereby authorized to charge the total fees indicated above to my deposit account.
- ☒ *(this check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.
- ☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

23-3030 (Deposit Account No.) | 18/5/99 (Date *(day/month/year)*) | Signature Jason J. SCHWARTZ, #43,910

See Notes to the fee calculation sheet

PCT INTERNATIONAL APPLIC[redacted] TRANSMITTAL LETTER	[redacted], May 1999
REGARDING THE INTERNATIONAL APPLICATION OF	DOCKET OR REFERENCE NUMBER
PURDUE RESEARCH FOUNDATION, et al	7024381Pur92
ENTITLED	
CALCIUM CHANNEL REGULATORS	

PCT/PTO 20 NOV 2000

09/700869

Certification under 37 CFR 1.10 (if applicable)

EM577549282US — "Express Mail" mailing number

18 May 1999 — Date of Deposit

I hereby certify that this application is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Leslie Curry

(Typed or printed name of person mailing application)

Leslie Curry

(Signature of person mailing application)

To the United States Receiving Office (RO/US):

Accompanying this transmittal letter is the above-identified International application, including a completed Request form (PCT/RO/101). Please process the application according to the provisions of the Patent Cooperation Treaty.

The following requests are made of the RO/US:

1. [XX] PREPARATION AND TRANSMITTAL OF CERTIFIED COPY OF PRIORITY DOCUMENTS—Please prepare and transmit to the International Bureau a certified copy of the United States origin priority documents identified in Box VI of the Request form (37 CFR 1.451).

   To cover the cost of copy preparation and certification (37 CFR 1.19(a)(3) and (b)(1)),

   [XX] a (check) (money order) in the amount of $ 30.00 included in fee is attached to this transmittal letter.

   [ ] the RO/US is hereby authorized to charge the following deposit account no.: _____.

2. [XX] CHOICE OF INTERNATIONAL SEARCHING AUTHORITY—It is requested that the International Search be performed by the following International Searching Authority:

   [XX] United States Patent and Trademark Office (ISA/US)

   [ ] European Patent Office (ISA/EP)

   The appropriate Search fee for the above-named Authority is indicated on the Fee Calculation Sheet (PCT/RO/101 Annex).

3. [XX] SUPPLEMENTAL SEARCH FEES (ONLY WHEN ISA/US CONDUCTS THE INTERNATIONAL SEARCH.)—Please charge any Supplemental Search fees that may be required by the United States International Searching Authority (ISA/US) to deposit account no.: 23-3030.

   I understand that this authorization is subject to my oral confirmation thereof in each instance and that it in no way limits my right to submit a protest against payment of the Supplemental Search fees, but is merely an administrative aid to assure that the ISA/US may timely complete the Search Report.

NOTE: SUPPLEMENTAL SEARCH FEES FOR ISA/EP ARE PAYABLE DIRECTLY TO THE EUROPEAN PATENT OFFICE

4. [XX] DISCLOSURE INFORMATION—In order to assist in screening the accompanying International application for purposes of determining whether a license for foreign transmittal should and could be granted and for other purposes, the following information is supplied:

   A. [ ] There is no prior filed application relating to this invention.

   B. [XX] There is a prior application, serial number 60/087,368 filed on 18 May 1998 (18.05.98) which contains subject matter that is and 60/098,072 filed on 27 August 1998 (27.08.98)

      1. [ ] substantially identical to that of the accompanying International application.

      2. [XX] less than that of the accompanying International application. The additional subject matter of the International application appears on pages(s) and line(s) throughout application.

      3. [ ] more than that of the accompanying International application.

   C. [ ] Disclosure information cannot be covered by the language of Points 4A or 4B above due to the involvement of several prior applications or for other reasons. A separate sheet on which the disclosure information is explained is attached to this transmittal letter.

5. [X] REQUEST FOR FOREIGN TRANSMITTAL LICENSE—According to the provisions of 35 U.S.C. 184 and 37 CFR 5.11, a license to transmit the accompanying International application to foreign agencies or international authorities is hereby requested.

SIGNER IS THE	NAME OF SIGNER (Typed)
[ ] APPLICANT	Jason J. Schwartz
[ ] COMMON REPRESENTATIVE	SIGNATURE
[X] (ATTORNEY) (AGENT) 43,910	
REG NO _____	

PTO-1382 (REV 3-84)
USCOMM DC 34-3817

U.S. Department of Commerce
Patent and Trademark Office

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 7024381Pur92

Box No. I TITLE OF INVENTION

CALCIUM CHANNEL REGULATORS

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

PURDUE RESEARCH FOUNDATION
Office of Technology Transfer
1063 Hovde Hall
West Lafayette, Indiana 47907 US

☐ This person is also inventor.

Telephone No. 765-494-2610

Facsimile No.

Teleprinter No.

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

PAK, William L.
1025 Windwood Lane
West Lafayette, Indiana 47906 US

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

SCHWARTZ, Jason J.
WOODARD, EMHARDT, NAUGHTON, MORIARTY & McNETT
Bank One Center/Tower, Suite 3700
111 Monument Circle
Indianapolis, Indiana 46204 US

SEE CONTINUATION TO BOX NO. IV ON SHEET NO. 4

Telephone No. 317-634-3456

Facsimile No. 317-637-7561

Teleprinter No. 810-341-3283

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

LI, Chenjian
221 Carrollwood Drive
Tarrytown, New York 10591

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: CN

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

GENG, Chaoxian
228 Arnold Drive
West Lafayette, Indiana 47906 US

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: CN

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☐ the United States of America only
- ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATE

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- ☒ AP **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ EA **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ EP **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ OA **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☒ AL Albania .....
- ☒ AM Armenia .....
- ☒ AT Austria .....
- ☒ AU Australia .....
- ☒ AZ Azerbaijan
- ☒ BA Bosnia and Herzegovina .....
- ☒ BB Barbados
- ☒ BG Bulgaria .....
- ☒ BR Brazil .....
- ☒ BY Belarus .....
- ☒ CA Canada
- ☒ CH and LI Switzerland and Liechtenstein
- ☒ CN China .....
- ☒ CU Cuba .....
- ☒ CZ Czech Republic .....
- ☒ DE Germany .....
- ☒ DK Denmark .....
- ☒ EE Estonia .....
- ☒ ES Spain .....
- ☒ FI Finland .....
- ☒ GB United Kingdom
- ☒ GD Grenada
- ☒ GE Georgia .....
- ☒ GH Ghana .....
- ☒ GM Gambia
- ☒ HR Croatia .....
- ☒ HU Hungary .....
- ☒ ID Indonesia
- ☒ IL Israel .....
- ☒ IN India .....
- ☒ IS Iceland
- ☒ JP Japan .....
- ☒ KE Kenya .....
- ☒ KG Kyrgyzstan .....
- ☒ KP Democratic People's Republic of Korea .....
- ☒ KR Republic of Korea .....
- ☒ KZ Kazakhstan .....
- ☒ LC Saint Lucia
- ☒ LK Sri Lanka
- ☒ LR Liberia
- ☒ LS Lesotho .....
- ☒ LT Lithuania
- ☒ LU Luxembourg
- ☒ LV Latvia
- ☒ MD Republic of Moldova .....
- ☒ MG Madagascar .....
- ☒ MK The former Yugoslav Republic of Macedonia .....
- ☒ MN Mongolia
- ☒ MW Malawi .....
- ☒ MX Mexico .....
- ☒ NO Norway
- ☒ NZ New Zealand .....
- ☒ PL Poland .....
- ☒ PT Portugal .....
- ☒ RO Romania
- ☒ RU Russian Federation .....
- ☒ SD Sudan
- ☒ SE Sweden
- ☒ SG Singapore
- ☒ SI Slovenia .....
- ☒ SK Slovakia .....
- ☒ SL Sierra Leone
- ☒ TJ Tajikistan .....
- ☒ TM Turkmenistan .....
- ☒ TR Turkey .....
- ☒ TT Trinidad and Tobago .....
- ☒ UA Ukraine .....
- ☒ UG Uganda .....
- ☒ US United States of America .....
- ☒ UZ Uzbekistan .....
- ☒ VN Viet Nam .....
- ☒ YU Yugoslavia .....
- ☒ ZW Zimbabwe .....

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- ☒ AE - United Arab Emirates .....
- ☒ ZA - South Africa .....
- ☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Supplemental Box *If the Supplemental Box is not used, this sheet should not be included with the request.*

*1. If, in any of the Boxes, **the space is insufficient** to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

*(i) **if more than two persons are involved as applicants and/or inventors** and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

*(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication **"the States indicated in the Supplemental Box"** is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, **the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America**: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) if, in addition to the agent(s) indicated in Box No. IV, there are **further agents**: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

*(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "**patent of addition**," or "**certificate of addition**," or if, in Box No. V, the name of the United States of America is accompanied by an indication "**continuation**" or "**continuation-in-part**": in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

*(vi) if, in Box No. VI, there are **more than three earlier applications whose priority is claimed**: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*

*(vii) if, in Box No. VI, **the earlier application is an ARIPO application**: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.*

*2. If, with regard to the **precautionary designation statement** contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

*3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning **non-prejudicial disclosures or exceptions to lack of novelty**: in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty" and furnish that statement below.*

Continuation to Box No. IV Agent

WOODARD, Harold R.; EMHARDT, C. David; NAUGHTON, Joseph A., Jr.; MORIARTY, John V.; McNETT, John C.; HENRY, Thomas Q.; DURLACHER, James M.; REEVES, Charles R.; WAGNER, Vincent O.; ZLATOS, Steve; BEREVESKOS, Spiro; BAHRET, William F.; BROWNING, Clifford W.; FRISK, R. Randall; LUEDERS, Daniel J.; GANDY, Kenneth A.; THOMAS, Timothy N.; SISSELMAN, Kerry P.; JONES, Kurt N.; ALLIE, John H.; BANTA, Holiday W.; COLE, Troy J.; PAYNTER, L. Scott; LOWES, J. Andrew; MEYER, Charles J.; HARRIS, Darrin Wesley; SCHANTZ, Matthew R.; COY, Gregory B.; HIDAY, Lisa A.; DANILUCK, John V.; BROWN, Christopher A.; SCHWARTZ, Jason J.; USHER, A. J., IV; COLLIER, Douglas A.; MYERS, James B. Jr.; STEVENS, Scott J., and ROWE, James L., all of Woodard, Emhardt, Naughton, Moriarty & McNett, Bank One Center/Tower, Suite 3700, 111 Monument Circle, Indianapolis, Indiana 46204 United States of America

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) (18.05.98) 18 May 1998	60/087,368	US		
item (2) (27.08.98) 27 August 1998	60/098,072	US		
item (3)				

XX The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): (1) and (2)

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA / US

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date (day/month/year)	Number	Country (or regional Office)
18 May 1998 (18.05.98)	60/087,368	US
27 August 1998 (27.08.98)	60/098,072	US

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets:**

request	:	5
description (excluding sequence listing part)	:	46
claims	:	8
abstract	:	1
drawings	:	14
sequence listing part of description	:	7
Total number of sheets	:	81

This international application is **accompanied by** the item(s) marked below:

1. [X] fee calculation sheet
2. [ ] separate signed power of attorney
3. [ ] copy of general power of attorney; reference number, if any:
4. [ ] statement explaining lack of signature
5. [ ] priority document(s) identified in Box No. VI as item(s):
6. [ ] translation of international application into (language):
7. [ ] separate indications concerning deposited microorganism or other biological material
8. [X] nucleotide and/or amino acid sequence listing in computer readable form
9. [X] other *(specify)*: Transmittal Letter

Figure of the drawings which should accompany the abstract: NONE

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

PURDUE RESEARCH FOUNDATION

(PAK, William L.)

(LI, Chenjian)

(GENG, Chaoxian)

Agent:

(Jason J. SCHWARTZ)

For receiving Office use only

1. Date of actual receipt of the purported international application:
2. Drawings: [ ] received: [ ] not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA /
6. [ ] Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

From the INTERNATIONAL BUREAU

PCT

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

SCHWARTZ, Jason, J.
Woodard, Emhardt, Naughton,
Moriarty & McNett
Bank One Center/Tower
Suite 3700
111 Monument Circle
Indianapolis, IN 46204
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 17 February 2000 (17.02.00)	
Applicant's or agent's file reference 7024381Pur92	**IMPORTANT INFORMATION**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US99/10821	18 May 1999 (18.05.99)	18 May 1998 (18.05.98)

Applicant
PURDUE RESEARCH FOUNDATION et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   AP :GH,GM,KE,LS,MW,SD,SL,SZ,UG,ZW
   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,BG,BR,CA,CN,CZ,DE,IL,JP,KP,KR,MN,NO,NZ,PL,RO,RU,SE,SK,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   EA :AM,AZ,BY,KG,KZ,MD,RU,TJ,TM
   OA :BF,BJ,CF,CG,CI,CM,GA,GN,GW,ML,MR,NE,SN,TD,TG
   National :AE,AL,AM,AT,AZ,BA,BB,BY,CH,CU,DK,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,
   ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MW,MX,PT,SD,SG,SI,SL,TJ,
   TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:
C. Cupello

Telephone No. (41-22) 338.83.38

09/700869 PCT FEB 03 2000

Woodard, Emhardt, Naughton Moriarty & McNett

To:

JASON J. SCHWARTZ
WOODARD, EMHARDT, NAUGHTON, MORIARTY &
MCNETT, BANK ONE CENTER/TOWER, STE. 3700
111 MONUMENT CIRCLE
INDIANAPOLIS IN 46204

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rule 59.3(e) and 61.1(b), first sentence and Administrative Instructions, Section 601(a))

Date of mailing *(day/month/year)*	31 JAN 2000
Applicant's or agent's file reference: 7024381PUR92	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/10821	18 MAY 99	18 MAY 98

Applicant: PURDUE RESEARCH FOUNDATION

1. The applicant is hereby **notified** that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

15 DEC 1999

2. That date of receipt is:

- [x] the actual date of receipt of the demand by this Authority (Rule 61.1(b)).
- [ ] the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).
- [ ] the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. [ ] **ATTENTION**: That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

- [ ] *(If applicable)* This notification confirms the information given by telephone, facsimile transmission or in person on:

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/US	Authorized officer
Assistant Commissioner for Patents Box PCT Washington, D.C. 20231 Attn: IPEA/US Facsimile No.	Catherine Williams PCT Team I Telephone No. (703) 305-3230 (FAX)

Form PCT/IPEA/402 (July 1998)

PATENT COOPERATION TREATY

RECEIVED
09/700869 JUL 31 2000

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

PCT Woodard, Emhardt, Naughton, Moriarty & McNett

To: JASON J. SCHWARTZ
WOODARD, EMHARDT, NAUGHTON, MORIARTY & MCNETT
BANK ONE CENTER/TOWER, SUITE 3700
111 MONUMENT CIRCLE
INDIANAPOLIS, INDIANA 46204

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	27 JUL 2000

Applicant's or agent's file reference	IMPORTANT NOTIFICATION
7024381Pur92	

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US99/10821	18 MAY 1999	18 MAY 1998

Applicant
PURDUE RESEARCH FOUNDATION

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	GABRIELE ELISABETH BUGAISKY
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)*

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 7024381Pur92	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US99/10821	International filing date *(day/month/year)* 18 MAY 1999	Priority date *(day/month/year)* 18 MAY 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant PURDUE RESEARCH FOUNDATION		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets.

   [X] This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I [X] Basis of the report
   - II [ ] Priority
   - III [ ] Non-establishment of report with regard to novelty, inventive step or industrial applicability
   - IV [ ] Lack of unity of invention
   - V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI [ ] Certain documents cited
   - VII [X] Certain defects in the international application
   - VIII [X] Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15 DECEMBER 1999	27 JUNE 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GABRIELE ELISABETH BUGAISKY Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (July 1998)*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/10821

I. Basis of the report

1. With regard to the elements of the international application:*

☐ the international application as originally filed

☒ the description:
pages 2-46 , as originally filed
pages 1 , filed with the demand
pages NONE , filed with the letter of _____

☒ the claims:
pages 47-54 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

☒ the drawings:
pages 1-14 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

☒ the sequence listing part of the description:
pages 1-7 , as originally filed
pages none , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☒ contained in the international application in printed form.

☒ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE
☒ the claims, Nos. NONE
☒ the drawings, sheets/fig NONE

5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	1-43	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	1-43	YES
	Claims	NONE	NO
Industrial Applicability (IA)	Claims	1-43	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 1-43 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest THE InaF gene, constructs containing the gene, a recombinant method of making InaF, and purified InaF protein. Proteins which regulate ion flux into cells are important in regulating signal transduction.

---------- NEW CITATIONS ----------

NONE

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

The description is objected to as containing the following defect(s) under PCT Rule 66.2(a)(iii) in the form or contents thereof: line 1 of the abstract recites "ulating". Presumably, this should be "modulating".

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to adequately enable practice of the claimed invention because: but a single gene is isolated which encodes an InaF protein that is involved in Ca^{2+} regulation and affects the levels of the TRP protein. The InaF gene is mapped to 10 C2-E3 of the *D. melanogaster* polytene chromosomes. Constructs containing the InaF gene are generated. The putative protein is 241 amino acids with an estimated weight of 26 kd and is recombinantly produced as a fusion partner to glutathione S-transferase. Although the description states that the invention is not limited to the specific sequences set forth in SEQ ID NO:1 and 2, and implies that other organisms possess a homologous protein with similar function, no evidence is presented that such proteins exist nor is it taught which specific positions could likely be mutated and still provide a protein of similar function. Similarly there is no evidence that a cognate gene exists in other organisms, no suggestion as to where such genes may likely be found. It is deemed that based upon a single working example of a gene and its encoded protein, that the description is not enabled for any homologue of the gene and/or protein, that insufficient identifying characteristics have been presented in order to one of skill in the art to determine whether a given gene/protein fulfills the same function of InaF. One has been given an invitation to experiment to try to identify and isolate other proteins/genes which may fall within the scope of the claims.

Claims 1, 3-4, 6, 8-9, 11-12, 14-16, 18, 20-21, 23, 25-26, 28-29, 30-34, 36-37, and 39-42 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the immediately preceding paragraph.

Applicants have responded to these objections by stating the description is replete with information to support the assertion that the description provides sufficient guidance to allow one skilled in the art to practice the invention without undue experimentation. It is stated that homologues of the described genes and proteins may be found by searching through various databases using computer programs known in the art or that portions of the nucleotide sequences may be used as probes to find similar sequences in various genomic and/or cDNA libraries. It is stated that other nucleotide sequences are taught in the application as the degeneracy of the genetic code allows one to alter the third base of a codon and achieve expression of the same amino acid. It is asserted that routine procedures exist in the art for determining whether a particular (Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): C07K 14/435; C12N 1/00, 5/10, 15/12, 15/63; C12P 21/00, 21/02 and US Cl.: 435/69.1, 320.1, 252.3, 325, 410; 530/350; 536/23.5

VIII. CERTAIN OBSERVATIONS ON THE APPLICATION (Continued):

polypeptide is involved in calcium ion regulation.

First, with respect to degenerate codon usage, there is no disagreement. It is regretted that it was not made sufficiently clear in the written opinion that the description is indeed enabled for alternate codon usage. However, but a gene encoding a single protein is described; the hybridizations to genomic DNA appear to be at high stringency and it is unclear whether the inaF gene is a member of a novel gene family so that multiple genes exist in *D. melanogaster*. There is no basis to conclude

that any nucleic acid molecule which encodes a protein of at least 30% identity to the disclosed inaF protein is indeed a genetic homologue of the disclosed inaF protein; other than the role of inaF in calcium regulation, the primary deduced sequence, and the chromosomal location of the gene, no other physical properties of the protein or gene are disclosed. An incomplete description of a single species does not *a priori* enable an entire genus. That a potential means exists for one to possibly obtain homologs of inaF does not in of itself show that the description is sufficiently enabling to allow one to unambiguously obtain these homologs.

CALCIUM CHANNEL REGULATORS

This invention was made with government support under grant number EY00033 awarded by the National Eye Institute. The Government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Patent Application Serial Number 60/087,368, filed on May 18, 1998, and U.S. Provisional Patent Application Serial Number 60/098,072, filed on August 27, 1998, both of which are hereby incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

In many types of excitable and nonexcitable cells, Ca^{2+} is both a critical molecule for homeostasis and an intracellular signaling molecule in many physiological processes such as muscle contraction, glandular secretion, transcriptional activation, and neurotransmitter release [Berridge, M.J. (1993) *Nature* 361: 315-325; Berridge, M.J. (1995) *Biochem. J.*, 312:1-11; Clapham, D.E. (1995) *Cell* 80:259-268; Clapham, D.E. (1996) *Neuron* 16:1069-1072]. Mobilization of Ca^{2+} is also involved in the immune response, such as autoimmune diseases and generation of an immune response after organ transplantation. Furthermore, a growing body of evidence suggests that neuronal degeneration diseases such as Alzheimer's is caused by excessive Ca^{2+} mobilization. These physiological processes are controlled by regulation of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). In resting cells, the cytosolic $[Ca^{2+}]_i$ is maintained at about 10-100 nM,

PATENT COOPERATION TREATY

09/700869

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

SCHWARTZ, Jason, J.
Woodard, Emhardt, Naughton,
Moriarty & McNett
Bank One Center/Tower
Suite 3700
111 Monument Circle
Indianapolis, IN 46204
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 13 August 1999 (13.08.99)	
Applicant's or agent's file reference 7024381Pur92	**IMPORTANT NOTIFICATION**
International application No. PCT/US99/10821	International filing date (day/month/year) 18 May 1999 (18.05.99)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 18 May 1998 (18.05.98)
Applicant PURDUE RESEARCH FOUNDATION et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
18 May 1998 (18.05.98)	60/087,368	US	12 July 1999 (12.07.99)
27 Augu 1998 (27.08.98)	60/098,072	US	22 June 1999 (22.06.99)

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From the INTERNATIONAL BUREAU

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
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Date of mailing (day/month/year) 25 November 1999 (25.11.99)		
Applicant's or agent's file reference 7024381Pur92		**IMPORTANT NOTICE**
International application No. PCT/US99/10821	**International filing date** (day/month/year) 18 May 1999 (18.05.99)	**Priority date** (day/month/year) 18 May 1998 (18.05.98)
Applicant PURDUE RESEARCH FOUNDATION et al		

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
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   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
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3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
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REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

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(51) International Patent Classification 6 :
C07K 14/435, C12N 1/00, 5/10, 15/12, 15/63, C12P 21/00

A1

(11) Internati nal Publication Number: **WO 99/60022**

(43) International Publication Date: 25 November 1999 (25.11.99)

(21) International Application Number: PCT/US99/10821

(22) International Filing Date: 18 May 1999 (18.05.99)

(30) Priority Data:

60/087,368	18 May 1998 (18.05.98)	US
60/098,072	27 August 1998 (27.08.98)	US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: CALCIUM CHANNEL REGULATORS

(57) Abstract

Purified InaF proteins that function in ulating calcium ion entry into cells are provided. Nucleotide sequences encoding functional InaF proteins are also provided. The invention also provides recombinant vectors including the nucleotide sequence encoding InaF, host cells that include the recombinant vectors described herein and methods of expressing InaF proteins by culturing such host cells.

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CALCIUM CHANNEL REGULATORS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Patent Application Serial Number 60/087,368, filed on May 18, 1998, and U.S. Provisional Patent Application Serial Number 60/098,072, filed on August 27, 1998, both of which are hereby incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

In many types of excitable and nonexcitable cells, Ca^{2+} is both a critical molecule for homeostasis and an intracellular signaling molecule in many physiological processes such as muscle contraction, glandular secretion, transcriptional activation, and neurotransmitter release [Berridge, M.J. (1993) *Nature* 361: 315-325; Berridge, M.J. (1995) *Biochem. J.*, 312:1-11; Clapham, D.E. (1995) *Cell* 80:259-268; Clapham, D.E. (1996) *Neuron* 16:1069-1072]. Mobilization of Ca^{2+} is also involved in the immune response, such as autoimmune diseases and generation of an immune response after organ transplantation. Furthermore, a growing body of evidence suggests that neuronal degeneration diseases such as Alzheimer's is caused by excessive Ca^{2+} mobilization. These physiological processes are controlled by regulation of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). In resting cells, the cytosolic $[Ca^{2+}]_i$ is maintained at about 10-100 nM,

but during stimulation the cytosolic $[Ca^{2+}]_i$ can rise rapidly to micromolar ranges.

The widely used signal transduction pathway, the receptor-based, G protein-coupled, PLC-IP3 cascade, also uses Ca^{2+} as a key signaling molecule. In excitable cells such as muscle cells, Purkinje neurons and *Drosophila* photoreceptor cells, as well as in nonexcitable cells such as mast cells and lymphocytes, extracellular stimuli activate receptors on the cell membrane, which in turn activate receptor-coupled G proteins. The activated G protein then activates phospholipase C to hydrolyze PIP_2 to IP3 and DAG. While DAG activates phosphokinases, IP3 binds to IP3 receptors, which are ligand-gated Ca^{2+} channels on the surface of intracellular Ca^{2+} stores, and induces Ca^{2+} release from these stores. The Ca^{2+} release from intracellular stores triggers, through unknown molecules and mechanisms, Ca^{2+} influx from the extracellular space into the cell via Ca^{2+} selective channels on the plasma membrane (reviewed by Berridge, 1995; Clapham, 1996, both cited above).

Putney, in *Cell Calcium* 11:611-624 (1990), proposed that activation of the Ca^{2+} channel on the plasma membrane is dependent on Ca^{2+} release from the intracellular stores, and named these specific types of Ca^{2+} channels on the plasma membrane "capacitative Ca^{2+} channels". In recent years, "capacitative Ca^{2+}

channels" has been renamed "store-operated Ca^{2+} channels (SOC)" because, unlike the capacitors in electronic circuitry, the Ca^{2+} channels on the cell membranes actually pass ions through them. Cells throughout the animal kingdom, as well as some bacterial, fungal and plant cells, have one or more types of calcium channels.

Although physiological and pharmacological studies identified the SOCs as a unique and important class of Ca^{2+} channels, no actual genes or proteins had been identified until the *Drosophila trp* gene was cloned and subsequently studied [Montell, C. and Rubin, G.M. (1989) *Neuron* 2:1313-1323; Wong, F.E.L. et al. (1989) *Neuron* 3:81-94; Hardie, R.C. and Minke, B. (1992) *Neuron* 8:643-651; Vaca, L. et al. (1994) *Am.J. Physiol.* 267:C1510-C1505]. Several lines of research have subsequently confirmed that the Drosophila Trp protein is a member of the SOCs. Since identification of the trp gene in Drosophila, several human and mouse homologs have been cloned [Wes, P.D. et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:9652-9656; Zhu, X. et al. (1995) *FEBS Letter* 373:193-198; Zitt, C. et al.(1996) *Neuron* 16:1189-1196]. Expression of the human Trp in COS cells increases store-operated calcium entry (SOCE), and expression of portions of mouse *trp* homologs in antisense orientation in murine L cells suppressed SOCE [Zhu, X. et al. (1995) above].

Further studies have determined that another protein, InaD, binds to the *Drosophila* Trp protein. InaD is a soluble protein with PDZ domains which are

known to be important for protein/protein interaction and anchoring ion channels [Kim, E. et al. (1995) *Nature* 378:85-88; Kim, E. et al. (1996) *Neuron* 17:103-113; Kormau, H.C. et al. (1995) *Science* 269:1737-1740]. InaD has been shown by co-immunoprecipitation and gel-overlay assays to bind physically to the trp protein (Shieh, B. and Zhu, M. (1996), *Neuron* 16:991-998; Huber, A. et al. (1996) *EMBO* 15(24);7036-7045]. It now appears that InaD forms the scaffold for a multimolecular signaling complex that includes the TRP protein. [Chevesich, J. et al. (1997) *Neuron* 18:95-105; Tsunoda, S. et al. (1997) *Nature* 388:243-249].

InaC has been identified as an eye-specific protein kinase C (Smith, D.P. et al. (1991) *Science* 254:1478-1484). InaC binds to InaD, suggesting that InaD could be one of the substrates of InaC-mediated phophorylation (Huber et al. (1996) above).

Although some information regarding regulation of calcium ion influx into a cell is known in Drosophila and higher eukaryotes, such as mice and humans, identification of other proteins involved in Ca^{2+} mobilization would increase the understanding of how calcium channels are regulated. Identification of proteins involved in calcium channel regulation in lower eukaryotes can lead to identification of similar proteins in higher eukaryotes, such as humans as discussed above for the trp protein. Moreover, identification of such proteins can lead to the identification of substances that modulate the activity of calcium channels, thus making it possible to treat diseases that are thought to involve calcium ion

mobilization, including Alzheimer's disease and autoimmune diseases. There is therefore a need for proteins and nucleic acid sequences involved in Ca^{2+} mobilization. The present invention addresses this need.

SUMMARY OF THE INVENTION

A novel protein, InaF, that functions in regulation of calcium ion entry into a cell, has been discovered. Accordingly, in one aspect of the invention, purified InaF proteins are provided.

In yet another aspect of the invention, isolated nucleic acid molecules that encode InaF proteins are provided. The nucleic acid molecules may be incorporated into a vector to form a recombinant nucleic acid molecule. Moreover, such recombinant nucleic acid molecules may be introduced into a host cell.

In other aspects of the invention, methods of expressing InaF proteins are provided. The methods include transforming a host cell with a nucleotide sequence encoding a protein that functions in regulating calcium ion entry into a cell as provided herein, and culturing the transformed host cells under conditions effective in achieving expression of InaF proteins. The proteins may then be purified by conventional techniques.

It is an object of the invention to provide purified functional InaF proteins.

It is a further object of the invention to provide nucleotide sequences encoding functional InaF proteins.

It is a further object of the invention to provide recombinant vectors that include nucleotide sequences encoding functional InaF proteins.

It is yet another object of the invention to provide host cells containing introduced nucleotide sequences encoding functional InaF proteins.

It is a further object of the invention to provide nucleotide sequences encoding functional InaF proteins and purified functional InaF proteins that may be modified to control calcium ion entry into cells.

These and other objects and advantages of the present invention will be apparent from the descriptions herein.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 depicts the cross scheme of single P local hopping mutagenesis for P69 and trol. Asterisks indicate chromosomes into which the P element could have transposed.

FIG. 2 depicts electroretinogram (ERG) recordings from inaF mutants as discussed in Example 2. The top trace is an ERG of strong allele, *inaF*^{P111x}, and the bottom trace is an ERG of weak allele, *inaF*^{P112x}. Stimulus duration was 4 seconds.

FIG. 3 depicts intracellularly recorded photoreceptor potentials as discussed in Example 2. The voltage responses to 8 second light stimuli were measured in wild-type flies, trp^{P301}, and inaF^{P111x}.

FIG. 4 depicts intracellularly recorded receptor potentials showing photoreceptor response latency as discussed in Example 2. Flies were dark-adapted for 2 minutes.

FIGS. 5A-B are views of photoreceptors obtained by transmission electron microscopy as discussed in Example 3. FIG. 5A, left panel, depicts photoreceptors of wild-type flies; FIG. 5A, right panel, depicts photoreceptors of 19 day old inaF;bw;st reared light/dark; FIG. 5B depicts an enlarged view of the region indicated by the arrow in FIG. 5A.

FIG. 6 depicts the cross-scheme for remobilization of the P insertion in $inaF^{P105p}$, as discussed in Example 4.

FIG. 7 depicts a cytogenetic map of the inaF mutation as discussed in Example 5. Df(1)HA85($inaF^-$), Df(1)m259-4($inaF^-$) and Df(1)($inaF^-$) are deficiency stocks as discussed in Example 5.

FIG. 8 depicts a genomic Southern analysis as discussed in Example 6. Genomic DNA was purified and digested by EcoRI (lanes 1-4), BamHI (lanes 5-8), and HindIII (lanes 9-12), and loaded on a 0.7% agarose gel in the following order: wild-type (lanes 1, 5 and 9); mutator 3B (lanes 2, 6 and 10); mutator 3B1-2 (jumpstarter)(lanes 3, 7 and 11); and $inaF^{P105p}$ (lanes 4, 8 and 12). The gel blot was probed with ^{32}P-dCTP labeled pCaSpeR3.

FIG. 9 depicts a genomic Southern analysis as discussed in Example 6. Genomic DNA was purified and digested by EcoRI (lanes 1 and 2), BamHI (lanes 3 and 4), and HindII (lanes 5 and 6), and loaded on a 0.7% agarose gel in the following order: mutator 3B (lanes 1, 3 and 5), and $inaF^{P105p}$ (lanes 2, 4 and 6).

FIG. 10 depicts a polytene chromosome after an in situ hybridization procedure performed as described in Example 7. The signal (arrowhead) detected on the polytene chromosome was localized in the 10 C2-E3 region of the X chromosome, which was consistent with

the results obtained by using pCaSpeR3 and fragment 4 of A23 as probes.

FIG. 11 depicts a Northern blot probed with cDNA #1 insert as discussed in Example 7. The lanes were loaded, from left to right, with polyA+ RNA from wild-type head, wild-type body, *inaF* (*inaF*^{P105P}) head and *eya* head. RP49, a ribosomal protein universally expressed in all tissues.

FIG. 12 depicts restriction maps of *inaF* cDNA and of the corresonding genomic region in the A23 clone and three inaF mutants. The unfilled inverted triangle in the *inaF*^{P105P} map identifies the P element insertion. The empty spaces to the right and left of the P insertion site in the *inaF*^{P106x} and *inaF*^{P111x} maps, respectively, represent the deletions caused by imprecise excision of the P element. In the cDNA map, the broken dotted line indicates the extent of the intron, and the open rectangle identifies the open reading frame. A composite genomic map at the top shows *Eco*RI sites (R) and the sizes of *Eco*RI fragments.

FIG. 13 depicts a Western blot analysis of null (*inaF*^{P106x}, *trp*^{P343}) and near-null (*inaF*^{P105P}, *trp*^{P301}) *inaF* and *trp* mutants, and wild-type and revertant controls. The seven lanes were loaded with total protein prepared from (lanes 1-7): wild-type heads, wild-type bodies, revertant heads, *trp*^{P301} heads, *trp*^{P343} heads, *inaF*^{P105P} heads, and *inaF*^{P106X} heads.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

For the purposes of promoting an understanding of the principles of the invention, reference will now be made to preferred embodiments and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations and further modifications of the invention, and such further applications of the principles of the invention as illustrated herein, being contemplated as would normally occur to one skilled in the art to which the invention relates.

A novel calcium channel regulator protein, InaF, has been identified in the fruit fly, *Drosophila melanogaster*. Accordingly, the present invention provides purified InaF protein. The invention further provides isolated nucleic acid molecules that include nucleotide sequences encoding functional InaF proteins. Recombinant nucleic acid molecules are also provided that include the novel inaF nucleotide sequence. The nucleic acid molecules may be incorporated in a host cell. In another aspect of the invention, methods of expressing functional InaF protein are also provided.

In a first aspect of the invention, novel, purified InaF proteins are provided that function in regulating cellular influx of calcium ions. The InaF polypeptides are substantially pure (i.e., InaF proteins are essentially free, e.g., at least about 95% free, from other proteins with which they naturally occur). In one preferred embodiment, the amino acid

sequence of an InaF protein, originally found in *Drosophila melanogaster*, is set forth in SEQ ID:1.

Although the invention is described with reference to *Drosophila melanogaster* amino acid sequences, it is understood that the invention is not limited to the specific amino acid sequences set forth in SEQ ID:1. Skilled artisans will recognize that, through the process of mutation and/or evolution, polypeptides of different lengths and having differing constituents, e.g., with amino acid insertions, substitutions, deletions, and the like, may arise that are related to, or sufficiently similar to, a sequence set forth herein by virtue of amino acid sequence homology and advantageous functionality as described herein. The term "InaF protein" is used to refer generally to a protein having the features described herein and a preferred example includes a polypeptide having the amino acid sequence of SEQ ID NO:1. Also included within this definition, and in the scope of the invention, are variants of the polypeptide which function in regulating calcium ion movement into a cell, as described herein.

It is well known that animals of a wide variety of species commonly express and utilize homologous proteins, which include the insertions, substitutions and/or deletions discussed above, and yet which effectively provide similar function. For example, an amino acid sequence isolated from another species may differ to a certain degree from the sequences set forth in SEQ ID NOS:1 and 2, and yet have similar functionality with respect to catalytic and regulatory

function. Amino acid sequences comprising such variations are included within the scope of the present invention and are considered substantially or sufficiently similar to a reference amino acid sequence. Although not being limited by theory, it is believed that the identity between amino acid sequences that is necessary to maintain proper functionality is related to maintenance of the tertiary structure of the polypeptide such that specific interactive sequences will be properly located and will have the desired activity. Although it is not intended that the present invention be limited by any theory by which it achieves its advantageous result, it is contemplated that a polypeptide including these interactive sequences in proper spatial context will have good activity, even where alterations exist in other portions thereof.

In this regard, an InaF protein variant is expected to be functionally similar to that set forth in SEQ ID NO:1, for example, if it includes amino acids which are conserved among a variety of species or if it includes non-conserved amino acids which exist at a given location in another species that expresses a functional InaF protein.

Another manner in which similarity may exist between two amino acid sequences is where a given amino acid of one group (such as a non-polar amino acid, an uncharged polar amino acid, a charged polar acidic amino acid or a charged polar basic amino acid) is substituted with another amino acid from the same amino acid group. For example, it is known that the uncharged polar amino acid serine may commonly be

substituted with the uncharged polar amino acid threonine in a polypeptide without substantially altering the functionality of the polypeptide. If one is unsure whether a given substitution will affect the functionality of the enzyme, then this may be determined without undue experimentation using synthetic techniques and screening assays known in the art.

The invention therefore also encompasses amino acid sequences similar to the amino acid sequences set forth herein that have at least about 30% identity thereto and function in regulating cellular influx of calcium ions. Preferably, inventive amino acid sequences have at least about 50% identity to these sequences, further preferably at least about 70% identity, more preferably at least about 80% identity and most preferably at least about 90% identity.

Percent identity may be determined, for example, by comparing sequence information using the advanced BLAST computer program, version 2.0.8, available from the National Institutes of Health. The BLAST program is based on the alignment method of Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 87:2264-68 (1990) and as discussed in Altschul, et al., *J. Mol. Biol.* 215:403-10 (1990); Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-7 (1993); and Altschul et al. (1997) *Nucleic Acids Res.* 25:3389-3402. Briefly, the BLAST program defines identity as the number of identical aligned symbols (i.e., nucleotides or amino acids), divided by the total number of symbols in the shorter of the two sequences. The program may be used

to determine percent identity over the entire length of the proteins being compared. Preferred default parameters for the BLAST program, blastp, include: (1) description of 500; (2) Expect value of 10; (3) Karlin-Altschul parameter λ = 0.270; (4) Karlin-Altschul parameter K = 0.0470; (5) gap penalties: Existence 11, Extension 1; (6) H value = $4.94e^{-324}$; (6) scores for matched and mismatched amino acids found in the BLOSUM62 matrix as described in Henikoff, S. and Henikoff, J.G. (1992) *Proc. Natl. Acad. Sci. USA* 89:10915-10919;Pearson, W.R. (1995) *Prot. Sci.* 4:1145-1160; and Henikoff, S. and Henikoff, J.G. (1993) *Proteins* 17:49-61. The program also uses an SEG filter to mask-off segments of the query sequence as determined by the SEG program of Wootton and Federhen (1993) *Computers and Chemistry* 17:149-163.

In another aspect of the invention, isolated nucleic acid molecules, originally isolated from *Drosophila melanogaster*, are provided that encode a functional InaF protein that functions in regulating calcium ion entry into cells. The nucleotide sequences are set forth in SEQ ID NOS:1 and 2. It is preferred that the nucleotide sequence includes nucleotides spanning nucleotides 301 to 1036 in SEQ ID NO:1 and nucleotides spanning nucleotides 528 to 1250 in SEQ ID NO:2. It is not intended that the present invention be limited to these exemplary nucleotide sequences, but include sequences having substantial similarity thereto and sequences which encode variant forms of functional InaF protein as discussed above and as further discussed below.

The term "isolated nucleic acid," as used herein, is intended to refer to nucleic acid which is not in its native environment. For example, the nucleic acid is separated from other contaminants that naturally accompany it, such as proteins, lipids and other nucleic acid sequences. The term includes nucleic acid which has been removed or purified from its naturally-occurring environment or clone library, and further includes recombinant or cloned nucleic acid isolates and chemically synthesized nucleic acid.

The term "nucleotide sequence," as used herein, is intended to refer to a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, including deoxyribonucleic acid and ribonucleic acid, and derivatives thereof. The terms "encoding" and "coding" refer to the process by which a nucleotide sequence, through the mechanisms of transcription and translation, provides the information to a cell from which a series of amino acids can be assembled into a specific amino acid sequence to produce a functional polypeptide, such as, for example, an active enzyme or other protein that has a specific function. The process of encoding a specific amino acid sequence may involve DNA sequences having one or more base changes (i.e., insertions, deletions, substitutions) that do not cause a change in the encoded amino acid, or which involve base changes which may alter one or more amino acids, but do not eliminate the functional properties of the polypeptide encoded by the DNA sequence.

It is therefore understood that the invention encompasses more than the specific exemplary nucleotide

sequence of *inaF*. For example, nucleic acid sequences encoding variant amino acid sequences, as discussed above, are within the scope of the invention. Modifications to a sequence, such as deletions, insertions, or substitutions in the sequence, which produce "silent" changes that do not substantially affect the functional properties of the resulting polypeptide molecule are expressly contemplated by the present invention. For example, it is understood that alterations in a nucleotide sequence which reflect the degeneracy of the genetic code, or which result in the production of a chemically equivalent amino acid at a given site, are contemplated. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product.

Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the encoded polypeptide molecule would also not generally be expected to alter the activity of the polypeptide. In some cases, it may in fact be desirable to make mutations in the sequence in order to study the effect of alteration on the biological activity of the

polypeptide. Each of the proposed modifications is well within the routine skill in the art.

In one preferred embodiment, the nucleotide sequence has substantial similarity to the sequence set forth in SEQ ID:1 or SEQ ID:2, preferably the sequence spanning nucleotides 314 to 1036 in SEQ ID:1 and preferably the sequence spanning nucleotides 528 to 1250 in SEQ ID:2, and variants described herein. The term "substantial similarity" is used herein with respect to a nucleotide sequence to designate that the nucleotide sequence has a sequence sufficiently similar to a reference nucleotide sequence that it will hybridize therewith under moderately stringent conditions. This method of determining similarity is well known in the art to which the invention pertains. Briefly, moderately stringent conditions are defined in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed. Vol. 1, pp. 101-104, Cold Spring Harbor Laboratory Press (1989) as including the use of a prewashing solution of 5X SSC (a sodium chloride/sodium citrate solution), 0.5% sodium dodecyl sulfate (SDS), 1.0 mM ethylene diaminetetraacetic acid (EDTA) (pH 8.0) and hybridization and washing conditions of 55°C, 5x SSC. A further requirement of the inventive polynucleotide is that it must encode a polypeptide having similar functionality to the InaF protein described herein, i.e., functioning to regulate influx of calcium ions into cells.

In yet another embodiment, nucleotide sequences having selected percent identities to specified regions of the nucleotide sequence set forth in SEQ ID:1 are

provided. In one preferred form, nucleotide sequences are provided that have at least about 60% identity, more preferably at least about 80% identity, and most preferably at least about 90% identity, to a nucleotide sequence of substantial length within the nucleotide sequence from nucleotides 314 to 1036 set forth in SEQ ID:1. For example, such length may be 100, 200 or 400 nucleotides, or may be the entire sequence from nucleotides 314 to 1036 of SEQ ID:1. A further requirement is that the nucleotide sequence from nucleotide 314 to 1036 set forth in SEQ ID:1 encodes a protein that functions in regulating calcium entry into cells. The percent identity may be determined, for example, by comparing sequence information using the advanced BLAST computer program, version 2.0.8., as described above with reference to amino acid identity. Preferred default parameters for blastn include: (1) Karlin-Altschul parameter λ = 1.37 (gapped and ungapped); (2) Karlin-Altschul parameter K = 0.711 (gapped and ungapped); (3)H = $4.94e^{-324}$ (gapped and zero for ungapped); (4) gap penalties: Existence 5, Extension 2; and (5) scores for matched and mismatched nucleotides found in the blastn matrix as described in Altschul, S.F. et al. (1997) *Nucleic Acids Res.* 25:3389-3402 and Zhang, J. (1997) *Genome Res.* 7:649-656.

A suitable DNA sequence may be obtained by cloning techniques using cDNA libraries. For example, *Drosophila melanogaster* head cDNA libraries are available commercially or may be constructed using standard methods known in the art. Suitable nucleotide

sequences may be isolated from DNA libraries obtained from a wide variety of species by means of nucleic acid hybridization or polymerase chain reaction (PCR) procedures, using as probes or primers nucleotide sequences selected in accordance with the invention, such as those set forth in SEQ ID:1, nucleotide sequences having substantial similarity thereto, or portions thereof.

Alternately, a suitable sequence may be made by techniques which are well known in the art. For example, nucleic acid sequences encoding a functional InaF protein may be constructed by recombinant DNA technology, for example, by cutting or splicing nucleic acids using restriction enzymes and DNA ligase. Furthermore, nucleic acid sequences may be constructed using chemical synthesis, such as solid-phase phosphoramidate technology. PCR may be used to increase the quantity of nucleic acid produced. Moreover, if the particular nucleic acid sequence is of a length which makes chemical synthesis of the entire length impractical, the sequence may be broken up into smaller segments which may be synthesized and ligated together to form the entire desired sequence by methods known in the art.

In another aspect of the invention, InaF polypeptides functioning in regulating calcium ion entry into a cell and having the amino acid sequences encoded by nucleotide sequences having substantial similarity to the nucleotide sequences described above are also provided.

In a further aspect of the invention, recombinant nucleic acid molecules, or recombinant vectors, are provided. In one embodiment, the nucleic acid molecules include a nucleotide sequence encoding a functional InaF protein. The nucleotide sequence has substantial similarity, as defined above, to the nucleotide sequence set forth in SEQ ID:1 or SEQ ID:2, preferably the sequence spanning nucleotides 314 to 1036 in SEQ ID:1 or the identical sequence in SEQ ID:2 spanning nucleotides 528 to 1250. The protein produced has the amino acid sequence set forth in SEQ ID:1, or variants thereof as described above.

Recombinant vectors may be constructed by incorporating the desired nucleotide sequence within a vector according to methods well known to the skilled artisan and as described for example, in Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Laboratory, Cold Springs Harbor, New York (1982). A wide variety of vectors are known that have use in the invention. For example, various plasmid and phage vectors are known that are ideally suited for use in the invention. For example, pGEM, pBluesript, EMBL and λGt11 may be used in the invention. In one embodiment, the desired recombinant vector may be constructed by ligating DNA linker sequences to the 5′ and 3′ ends of the desired nucleotide insert, cleaving the insert with a restriction enzyme that specifically recognizes sequences present in the linker sequences and the desired vector, cleaving the vector with the same restriction enzyme, mixing the cleaved vector with

the cleaved insert and using DNA ligase to incorporate the insert into the vector as known in the art.

The vectors may include other nucleotide sequences, such as those encoding selectable markers, including those for antibiotic resistance or color selection. The vectors also preferably include a promoter nucleotide sequence. The desired nucleic acid insert is preferably operably linked to the promoter. A nucleic acid is "operably linked" to a another nucleic acid sequence, such as a promoter sequence, when it is placed in a specific functional relationship with the other nucleic acid sequence. The functional relationship between a promoter and a desired nucleic acid insert typically involves the nucleic acid and the promoter sequences being contiguous such that transcription of the nucleic acid sequence will be facilitated. Two nucleic acid sequences are further said to be operably linked if the nature of the linkage between the two sequences does not (1) result in the introduction of a frame-shift-mutation; (2) interfere with the ability of the promoter region sequence to direct the transcription of the desired nucleotide sequence, or (3) interfere with the ability of the desired nucleotide sequence to be transcribed by the promoter sequence region. Typically, the promoter element is generally upstream (i.e., at the 5' end) of the nucleic acid insert coding sequence.

A wide variety of promoters are known in the art, including cell-specific promoters, inducible promoters, and constitutive promoters. The promoters may further be selected such that they require activation by

activating elements known in the art, so that production of the protein encoded by the nucleic acid sequence insert may be regulated as desired.

The vectors may further include other regulatory elements, such as enhancer sequences, which cooperate with the promoter to achieve transcription of the nucleic acid insert coding sequence. By "enhancer" is meant nucleotide sequence elements which can stimulate promoter activity in a cell, such as a bacterial or eukaryotic host cell.

Moreover, the vectors may include another nucleotide sequence insert that encodes a protein that may aid in purification of the desired protein encoded by the desired nucleotide sequence. The additional nucleotide sequence is positioned in the vector such that a fusion, or chimeric, protein is obtained. For example, an InaF protein may be produced having at its C-terminal end linker amino acids, as known in the art, joined to the other protein. The additional nucleotide sequence may include, for example, the nucleotide sequence encoding glutathione-S-transferase (GST). After purification procedures known to the skilled artisan, the additional amino acid sequence is cleaved with an appropriate enzyme. For example, if the additional amino acid sequence is that of GST, then thrombin is used to separate the InaF protein from GST. The InaF protein may then be isolated from the other proteins, or fragments thereof, by methods known in the art.

The inventive recombinant vectors may be used to transform a host cell. Such methods include, for

example, those described in Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Laboratory, Cold Springs Harbor, New York (1982). Once the desired nucleic acid has been introduced into the host cell, the host cell may produce the inventive InaF protein, or variants thereof, as described above. Accordingly, in yet another aspect of the invention, a host cell is provided that includes the inventive recombinant vectors described above.

A wide variety of host cells may be used in the invention, including prokaryotic and eukaryotic host cells. Bacterial host cells such as *Escherichia coli*, HB 101 and XL-1 blue may be advantageously used in the present invention. Typical eukaryotic host cells include SF9, S2, NIH 3T3 and NIH 293.

In yet another aspect of the invention, methods of producing functional InaF proteins as described above are provided. In one embodiment, the method includes providing a nucleotide sequence described above, or variants thereof, that encodes a functional InaF protein that regulates calcium ion entry into cells, and introducing the nucleotide sequence into a host cell, as described above. The desired nucleotide sequence may be advantageously incorporated into a vector to form a recombinant vector. The recombinant vector may then be introduced into a host cell according to known procedures in the art. Such host cells are then cultured under conditions, well known to the skilled artisan, effective to achieve expression of the InaF polypeptide. The InaF polypeptide may then be purified using conventional techniques.

Reference will now be made to specific examples illustrating the invention described above. It is to be understood that the examples are provided to illustrate preferred embodiments and that no limitation to the scope of the invention is intended thereby.

EXAMPLE 1

Generation of inaF Mutant by P-Element Mediated Mutagenesis

This example shows the method by which *inaF* mutants were obtained through P-element mediated mutagenesis.

Drosophila Stocks

The first *inaF* mutant was generated through P-element mediated mutagenesis, as described below, on a *white*$^-$ background. The actual eye color of the mutant was light orange because the P element insertion causing the *inaF* mutation contains a *mini-white*$^+$ gene. To eliminate eye color, the original *inaF* mutant was placed in a *bw; st* background, so that *inaF; bw; st* flies would have no eye-color pigment.

In accordance with the Pak laboratory's practice of giving a P1XX number to 1st chromosome mutations and using a lower-case letter after the number to indicate the method of inducing the mutations, we designated the original *inaF* as $inaF^{P105p}$, in which the lower-case p in the superscript stands for P-insertion. In the course of a remobilization experiment to be described below, 25 new *inaF* alleles were generated due to imprecise excision. For these additional alleles we used a lower-case x in the superscript to indicate that

they were induced by imprecise excision and designated the 25 new alleles in as *inaF*^{P106x} through *inaF*^{P130x}.

The mutator, 3B, was chosen for local hopping mutagenesis, because it has an insertion in 3B1-2, which is very close to 3A3-5 where the *P69* gene is localized. This fly has a mutation in an eye-pigment gene *white*, and thus originally has a white eye color background. The actual eye color of 3B is orange, because the fly also carries a P element, pCaspeR3, which has the mini-*white*$^{+}$ gene as a marker. The shades of eye color, from dark red to light lemon, are dependent on where the insertions are. The eye color is darker when the insertion is in the vicinity of a strong enhancer, and the eye color is lighter when the insertion is close to a weaker enhancer. This location-sensitive eye color change is a very good indication of whether the P element has been mobilized to a new place.

The mutator, y w/P[lacW] was chosen for random targeting mutagenesis. This fly carries a *white*$^{-}$ mutation, and thus has a white eye color background. Its actual orange eye color is from the P[lacW], an engineered P element with the plasmid rescue feature as well as the enhancer trap capability as described in Bier et al., *Genes and Development*, 3:1273-1287 (1989).

The jumpstarter, *P3629*, carries a functional transposase gene which lacks the end inverted repeats (delta2-3). The delta2-3 is inserted on the 3rd chromosome, which also carries a visible dominant marker Sb. This visible marker is useful in indicating the presence or absence of the delta2-3.

The *trol* mutation is lethal. In the *trol* stock used in this mutagenesis, females are balanced over FM7, and males carry, by translocation, a 2D-3C segment of the X chromosome on the Y chromosome, which rescues the *trol* lethality.

In the *C(1)RM, y w/w/Y* fly stock, females have a special type of genome that contains two linked X chromosomes. These two linked chromosomes will segregate together. If a male fly is mated to *C(1)RM, y w/Y* females, all the male offspring will carry the same X chromosome as the P1 male.

Local hopping mutagenesis to target *trol/P69*

The P element mediated local hopping mutagenesis was undertaken with the aim of isolating lethal *trol* alleles or viable ERG mutants (FIG. 1). There were three generations of crosses before the mutagenesis result could be tested by ERG.

Cross I: The mutator, 3B, which carries a pCaspeR3 in *white*$^-$ background was used. Its eye-color is light orange. The jumpstarter stock carries delta2-3 on the third chromosome, which is marked with a dominant marker Sb. In each bottle, 20 mutator males and 20 jumpstarter females were combined. Parent flies in each bottle were transferred after 4-5 days to a new bottle once, and then discarded. All flies were raised at 25^{o}C.

Cross II: Among the progeny of cross I, virgin females with Sb marker were selected to mate with males

from a FM0 containing stock. The females carried both the mutator on the X chromosome, and the jumpstarter on the 3rd chromosome. In the germ line cells of these flies, the P element in the mutator could be mobilized to new chromosomal locations because of the transposase activity conferred by delta2-3. In each round of the mutagenesis, 20 bottles of cross II were set up, each containing 20 virgin females and 20 males. Parent flies were transferred once to new food after 4-5 days, and then discarded. All flies were raised at 25°C.

Cross III: From the progeny of cross II, flies were selected for remobilized pCaspeR3, by selecting for flies with changed eye color shades (presence of *white*$^{+}$). Flies were also selected against Sb-marked delta2-3, so that pCaspeR3 insertions would be stabilized.

Both male and female progeny of cross II were used:

1) Males were single-mated to *C(1)RM, y w/Y* females carrying an attached X chromosome to establish stable lines. After 7 days, male parents were scored by an electroretinogram (ERG) as described in Example 2. If the ERG showed a mutant phenotype, the line was saved for further study; and if the ERG were wild type, the line was discarded.

2) Virgin females were single-mated to *trol/W*$^{+}$*Y* males. After 7 days, parents were transferred to fresh food. The offspring of this single-female-mating have four possible genotypes, indicated as A,B,C and D in FIG. 1.

If type A flies were found, the line was discarded because the insertion obviously was not into the *trol* gene. If type A flies were not found, then the P insertion was in the *trol* gene. The D type flies were saved for further study.

Analysis

Three rounds of local hopping mutagenesis were performed. Cross II yielded about 2% of offspring that showed changes of eye color, indicating that the P element was mobilized to new chromosomal locations. Approximately $2X10^4$ F_2 flies were scored.

Virgin female F_2 flies with eye color changes were single-female-mated to *trol*/W^+Y. The offspring of this cross were scored for complementation with *trol*. Among 179 such single-female-mating lines, none was identified as a *trol* allele.

Male F_2 flies with eye color changes were single male mated to C(1) RM, y w/Y. Among the offspring of this single-male-mating, all males carried the same X chromosome as the single male parent. 1-2 males of each line were scored by ERG. In 255 such single male mating lines, one was identified as a new mutant and designated as *inaF*.

EXAMPLE 2

Electrophysiological Identification of *inaF* Phenotypes

Electroretinogram

The electroretinogram (ERG) is an extracellular measurement of the light-induced responses in the eyes. The ERGs were recorded as described in Pak, W.L. et al.

Nature 222:351-354 (1969). A xenon arc lamp (Oriel) served as the light source with an infrared filter (7CS1-75, Corning) and Wratten neutral density filters (Kodak) were used to modulate its intensity and infrared content. In most cases, flies were raised to 5 day post-eclosion for ERG recordings. In the case of the *P69;bw;st trp*^{CM} double mutant, however, 1 day old flies were used because photoreceptors in the double mutant showed massive degeneration by day 5, but no visible defects in the eye structure on day 1.

Intracellular Recording

The intracellular recording technique was performed as described in detail by Johnson, E.C. et al. (1986) *J. Gen. Physiol.* 88(5):651-673. Flies anaesthetized with CO_2 were mounted on a glass coverslip with myristic acid. A small portion (<10%) of the cornea was cut off with a vibrating razor blade. A thin layer of inert vacuum grease was applied to the cut end to prevent desiccation of the retina.

Both the reference and the recording electrodes were inserted into the eye through the cut end of the cornea. The reference electrode was a low resistance glass microelectrode filled with physiological saline and was placed into the retinal layer of the eye. The intracellular recording electrode (FHC Borosil 1.2 mm) was pulled on a vertical Narashige puller, filled with 2 M KCl, and selected for resistance ranging between 30 to 100 mega ohm. The recording electrode was inserted into the retinal layer with a Leitz micromanipulator. Penetration of a photoreceptor was done by a minute

forward movement of the electrode and a simultaneous delivery of a brief overdriving negative capacitance current to induce oscillation at the tip of the electrode. Successful penetration of a photoreceptor cell was indicated by a drop in voltage of more than 30 mV as seen on the oscilloscope and a receptor potential of more than 20 mV in response to a bright light stimulus. The preparation was dark adapted for more than 2 minutes before any further experiments.

The measured voltage was fed to a WPI preamplifier from which the signals were directed to both an oscilloscope and a digitizer (Digidata 1200, Axon Instrument). The digitized signals were filtered at 100 Hz and were recorded by Axoscope in a Pentium computer.

Analysis

In the study of *inaF*, all flies for ERG were 2-4 days posteclosion. The most obvious mutant phenotype of *inaF* revealed by ERG and intracellular recordings is that the receptor potential fails to maintain a steady-state response during light stimuli and decays rapidly toward base line (FIGS. 2 and 3).

The rate of decay was allele-dependent. Strong alleles such as $inaF^{P111x}$ caused the receptor potential to decay to base line within 4-5 seconds under bright light stimuli. Intermediate alleles such as $inaF^{P112x}$ caused slower decay, and some of them never caused complete decay to the base line even under bright light stimuli.

The rate of decay was also dependent on the light intensity (FIG. 2) and was faster under brighter light in all *inaF* alleles.

This receptor potential decay in *inaF* closely resembles the phenotype displayed by the *trp* mutant. When the strongest mutant alleles of the two genes, $inaF^{P111x}$ and trp^{P301}, were compared, $inaF^{P111x}$ caused a stronger mutant phenotype in speed and extent of receptor potential decay (FIG. 3). FIG. 3 also shows that the receptor potential of wild-type flies is maintained at a steady state.

Another mutant phenotype became evident when the latency between the light stimulus and the photoreceptor response was examined (FIG. 4). The latency is defined as the delay between the onset of the light stimulus and the beginning of photoreceptor depolarization. This delay is light intensity dependent and has been interpreted as the time required by the phototransduction pathway to proceed from photoconversion of rhodopsin to the opening of light-activated channels on the plasma membrane. In both trp^{P301} and $inaF^{P111x}$, the latency was prolonged compared to that of the wild type, and the delay was greater in $inaF^{P111x}$.

EXAMPLE 3

Effect of inaF Mutation on Retinal Degeneration

This example shows that the *inaF* photoreceptors undergo a light-dependent degeneration. Degeneration is also age-dependent and is not detectable in young (<1 week old) inaF mutants.

Transmission Electron Microscopy

The transmission electron microscopy technique was identical to the method described by Fan, S.S. and Ready, D.F. (1997) *Development* 124:1497-1507. Flies were microinjected with aldehyde fixative (2% paraformaldehyde and 1.75% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4) and dissected after 1 hour. Fixed eyes were incubated in 1% tannic acid overnight and transferred to 2% osmium tetroxide in 0.1 M sodium cacodylate for 2 hours. After washing, the eyes were incubated overnight in 2% uranyl acetate. After a serial dehydration with ethanol, eyes were mounted in Epox 812. Tissue was then sectioned using a Reichert ultramicrotome and observed using a Philips 300 electron microscope.

Analysis

Retinal degeneration was observed in *inaF* compound eyes (FIGS. 5A-B). For better control, $inaF^{P105p}$ was put in a *bw; st* background to eliminate eye color. Confocal microscopy and EM were used to examine photoreceptor structures. The photoreceptors in $inaF^{P105p}$*; bw; st* showed no detectable abnormality at 1 day posteclosion, suggesting that they had developed normally. However, flies raised in a 12 hour-light/12 hour-dark cycle to 19 days posteclosion showed retinal degeneration. Rabdomeres were absent in some ommatidia; most of the rabdomeres were much reduced in size; microvilli (membrane which contains rhodopsin) were disrupted by vacuolized structures; and the base of the microvilli was no longer smooth and regular.

To test if this retinal degeneration was light dependent, *inaF*^{P105p}; *bw; st* flies were raised in complete darkness from the embryo stage to 19 days posteclosion. EM study of these fly eyes indicated that the photoreceptor structure was largely intact (data not shown). Hence the retinal degeneration in *inaF* is light dependent.

EXAMPLE 4

Proof that the inaF Mutation is Caused by P-Element Insertion - Remobilization of the P insertion in *inaF*^{P105p}

The most direct and reliable method to demonstrate that a mutation is in fact caused by a P element insertion is to remobilize the P element. If the P element is the cause of the mutation, then one would expect two possibilities when the P insertion is removed from the genome. First, if the P element is precisely excised, then the mutated gene will be restored, and the mutant flies revert to wild type (thus they are called "revertants"). Second, if the excision is imprecise and takes away some flanking DNA with the remobilized P element, the flies will continue to show a mutant phenotype.

If, however, the mutation is actually not caused by the P insertion, but by some other defect such as a spontaneous point mutation, then the flies with the P element remobilized will always remain mutant, i.e., no wild-type revertants will be recovered.

Remobilization of the P insertion in *inaF*^{P105p}

inaF^{P105p} was used as a mutator and crossed to the jumpstarter, *P3269*. The design and protocol for this remobilization experiment are the same as those of local hopping mutagenesis described in Example 1 above, except that white-eyed flies (indicating loss of the P element which carries the *mini-white*$^{+}$ gene) were selected among the offspring of cross II (FIG. 6). These virgin females were single mated to FM0/Y males. The offspring had four possible genotypes (X- indicates an excision event):

A: X- *inaF*^{P105p} -X/FM0; +/+; +/+

B: FM0/FM0; +/+;+/+

C: FM0/Y;+/+;+/+

D: X- *inaF*^{P105p} -X/Y;+/+;+/+

Type D offspring in each single-female line were selected for ERG. If the ERG showed the *inaF* phenotype, the excision event was an imprecise one. Types A and D were saved to establish stable lines of new *inaF* alleles. If the ERG showed a wild-type response, the excision event was a precise one. Types A and D were saved to establish stable lines of these revertants. These lines were used in chromosomal *in situ* hybridization with the P element as a probe to confirm that the P insertion in *inaF*^{P105p} no longer existed.

Analysis

In cross III, 260 single-female-mating lines were set up, and their offspring were scored by ERG. We obtained 126 wild-type revertants, 61 lethal mutants, and 25 mutants showing the *ina* ERG phenotype and thus presumed to carry new mutant *inaF* alleles. Flies from these 25 lines were crossed to $inaF^{P105p}$ for complementation and confirmed to carry mutant alleles of $inaF^{P105p}$.

This result unequivocally demonstrated that the $inaF^{P105p}$ mutation is caused by a P element insertion.

EXAMPLE 5

Cytogenetic Mapping of the $inaF^{P105p}$ Mutation

To map the *inaF* mutation cytogenetically, a group of deficiency stocks were obtained from the *Drosophila* Stock Center at Indiana University and mated to the *inaF* mutant. The heterozygous F_1 flies that carried Deficiency/*inaF* chromosomes were scored by ERG.

Analysis

A group of deficiency stocks carrying deletions in the 10 C2-E3 region were used to map the $inaF^{P105p}$ mutation cytogenetically (FIG. 7). Three of them did not complement the $inaF^{P105p}$ mutation. Thus, results from cytogenetic mapping independently localized the $inaF^{P105p}$ mutation to the 10 C2-E3 region of the X chromosome, consistent with the P insertion site

identified by chromosomal *in situ* hybridization as described in Example 9 below.

EXAMPLE 6

An Eye-Specific Clone A23 Fragment Contains the inaF Gene

This example shows that, by analyzing genomic Southern and Northern blots, clone A23 was shown to contain the *inaF* structural gene.

Previous research has shown that the majority of genes important for phototransduction are expressed specifically or preferentially in the eyes. Since *inaF* is a vision defective mutant and clone A23 co-localizes with *inaF*, it is possible that clone A23 may contain the *inaF* structural gene. However, the 10 C-D-E region of the X chromosome contains about 500 kb of genomic DNA which accommodates about 50-100 genes. Therefore it is also possible that clone A23 represents an eye-specific gene in that region but is unrelated to the *inaF* gene. This question was resolved by a combination of genomic Southern and Northern analyses.

Isolation of Clone A23

Several years ago, the Pak laboratory isolated a pool of *Drosophila* eye-specific clones by subtractive hybridization. In that method, poly(A)$^+$ RNA extracted from the heads of wild type flies was reverse transcribed into cDNA and hybridized with an excess amount of poly(A)$^+$ RNA extracted from the heads of *eyes absent* (*eya*) mutant flies, all according to standard

protocols. The eye-specific, single-stranded cDNA molecules were then separated from the hybridization mixture by hydroxyapatite chromatography according to standard protocols and used to screen a genomic library to generate a pool of eye-specific clones. These clones were further confirmed by dot blots and Northern blots. The confirmed eye-specific clones were localized on the polytene chromosomes by chromosomal *in situ* hybridization. One of them, A23, was localized in the 10 D region of the X chromosome.

Genomic Southern Analysis

Genomic DNA of wild-type flies, the 3B mutators and the $inaF^{P105p}$ flies was isolated by homogenizing fifteen to twenty flies and using the Puregene kit from Gentra Co. following recommended protocols. 3 µg of genomic DNA of each type was digested with restriction enzymes of choice and loaded on a 0.7% agarose gel for electrophoresis. The agarose gel was denatured in 1.5 M NaCl, 0.5 M NaOH solution for 30 minutes, neutralized in 1 M Tris-Cl, 3 M NaCl, pH 7.5 solution for 40 minutes, blotted overnight onto Hybond-N^+ Nylon membrane (Amersham Co.), and UV cross-linked.

1 µg of genomic or cDNA fragments was used as template for ^{32}P-dCTP labeling with random primers. The radioactively labeled probe was purified with a Sephadex G-50 column.

Prehybridization treatment was carried out in 0.5 M NaH_2PO_4, 0.7% SDS, 1% BSA, 0.01 M EDTA solution at 68°C for 3-4 hours, and hybridization was carried out in the same solution at 68°C for 16-20 hours. Washing was carried out in 0.04 M sodium phosphate buffer, 5%

SDS, 0.5% BSA, 0.01 M EDTA solution twice for 20 minutes and in 0.04 M sodium phosphate buffer, 1% SDS, 0.01 M EDTA solution twice for 40 minutes. Kodak X-ray film was used for autoradiography.

Northern Analysis

The poly(A)$^+$ RNA was extracted with a PolyATtract-1000 kit from Promega Co. following their recommended protocol. 3 µg of poly(A)$^+$ RNA was loaded in each lane of the agarose gel unless otherwise specified. 1 µg of genomic DNA or cDNA fragment was used as template for ^{32}P-dCTP labeling with random primers. Prehybridization, hybridization, and washing of Northern blots were carried out according to the standard protocol in Sambrook et al., *Molecular Cloning A Laboratory Manual*, 2nd ed. Vol. 1, Cold Spring Harbor Laboratory Press (1989).

Analysis

Genomic Southern analyses were used to determine whether A23 contains DNA fragments flanking the P element insertion that causes the *inaF* mutation. Since the P insertion is in *inaF*, A23 could not contain the *inaF* gene if it were far removed from the P insertion.

Genomic DNA from wild-type flies, mutator 3B, and *inaF*^{P105p} was purified and digested with multiple restriction enzymes, electrophoresed and blotted. A genomic Southern blot was probed with pCaSpeR3 (FIG. 8). Restriction fragment length polymorphism (RFLP) was observed and can be interpreted as follows: 1) The RFLP between wild type and 3B is due to an additional P element in 3B; and 2) The RFLP between 3B and

inaF^{P105p} is due to the fact that DNA fragments of different sizes flank the P element insertion sites in 3B and *inaF*, and these were detected by the ^{32}P-dCTP labeled pCaSpeR3 probe.

Other Genomic Southern blots were probed by ^{32}P-dCTP labeled A23 fragments. Among A23 fragments, fragment 4 (3.6 kb) detected RFLPs between wild-type flies, 3B, and *inaF*^{P105p} that were similar to those detected by the pCaSpeR3 probe as seen in FIG. 8, except that the EcoRI lanes showed same size signals. This could be due to the fact that the new flanking DNA sequences in *inaF*^{P105p}, though a different species, has the same size as the one flanking the P insertion in 3B. The similarity of the RFLPS between wild-type flies, 3B, and *inaF*^{P105p} indicates that fragment 4 is likely to contain DNA flanking the P insertion site in *inaF*^{P105p} (FIG. 9).

Northern blots were used to examine whether fragment 4 of A23 could detect alterations of transcripts between wild-type flies and *inaF*^{P105p}. Poly(A)$^+$ RNA was purified from wild-type fly heads, wild-type fly bodies, *eya* heads, and *inaF*^{P105p} heads. Because *inaF*^{P105p} flies undergo age-dependent retinal degeneration, and because confocal microscopy did not detect retinal degeneration in young (<5 days) *inaF*^{P105p} flies, polyA$^+$ RNA was purified from 1-3 days old *inaF*^{P105p} flies. Fragment 4 of A23 was used as a probe for Northern analysis, and detected a 3.0 kb eye-

specific transcript which was drastically reduced in *inaF*^{P105p} flies.

Results from the genomic Southern and Northern analyses jointly indicated that fragment 4 of clone A23 contained at least part of the inaF gene and possibly all of it.

EXAMPLE 7

Isolation of inaF cDNA Clones From a *Drosophila* Head cDNA Library

Screening Procedure

Fragment 4 of A23, a genomic DNA fragment, was used as a template for ^{32}P-dCTP labeling with random primers. The labeled probe was purified with a Sephadex G-50 column and was used to screen 5 X 10^5 plaque forming units (pfu) of a *Drosophila* head cDNA Library, a gift from Dr. Erich Buchner at Wurzburg University in Germany. The cDNA library screening was carried out according to a standard protocol in Sambrook et al., *Molecular Cloning A Laboratory Manual*, 2nd ed. Vol. 1, Cold Spring Harbor Laboratory Press (1989).

Analysis

10 positively hybridizing cDNA clones were obtained and purified as single plaques. Cross hybridization among these clones demonstrated that they all belong to the same class of cDNA. cDNA#1 had the biggest insert and thus was used for further experiments. The insert of cDNA#1 was labeled with

biotin-dUTP and used as a probe for chromosomal *in situ* hybridization and detected a hybridization signal in the 10 C2-E3 region as described in Example 8. The insert was also used to probe a genomic Southern blot and detected the same RFLP as those revealed by pCaSpeR3 and A23 probes. Finally, the insert was labeled with ^{32}P-dCTP and used to probe a Northern blot.

Three μg of polyA+ RNA of each sample was loaded on the gel. cDNA#1 insert was labeled with ^{32}P-dCTP. A 3.0 kb transcript was detected in the poly(A)$^+$ RNA from wild-type fly heads but not that from wild-type fly bodies and *eya* heads, indicating that the 3.0 kb transcript is eye specific (FIG. 11). The same transcript was absent from the poly(A)$^+$ RNA from inaF^{P105p}, indicating that the cDNA most likely contains the inaF gene. The same blot was boiled to eliminate the radioactive probe and used again for a control experiment in which RP49, a ribosomal protein universally expressed in all tissues, was used as a probe. These lines of evidence all suggested that cDNA#1 corresponds to the *inaF* gene.

EXAMPLE 8

Chromosomal Location of the *inaF^{P105p}* P Insertion

To localize the *inaF^{P105p}* P insertion site on polytene chromosomes, pCaSpeR3, the P element employed in the local hopping mutagenesis described in Example 1, was used as a template for synthesizing biotin-dUTP probes for chromosomal *in situ* hybridization to the

polytene chromosomes of *inaF*^{P105p}. In this case, however, a genomic DNA fragment from clone A23, discussed in Example 6, and a cDNA fragment of cDNA#1 were used as templates for the biotin-dUTP labelling. The probe hybridized to 10 C2-E3 (FIG. 10). The signal at 10 C2-E3 was due to detection of the new P insertion.

EXAMPLE 9

Sequence of *inaF* cDNA

Sequencing cDNA clone #1

cDNA clone #1 was partially digested with EcoRI and subcloned into the pBluescript-SK$^+$ vector. T3 and T7 primers were used for initial sequencing reactions, and internal sequencing primers were designed and synthesized according to the sequence data obtained from each gel reading. The sequencing reactions were carried out at the DNA Sequencing Center at Iowa State University, Ames, Iowa. Both strands were sequenced, and every nucleotide has been confirmed from at least three independent reactions. The sequence of cDNA clone 1 is set forth in SEQ ID:1. A similar nucleotide sequence, differing only in certain 5' regions, and including a linker sequence at the 3' end, is set forth in SEQ ID:2.

Analysis

The cDNA has a poly(A)$^+$ tail immediately before the 3'-end EcoRI cloning site, and a consensus

polyadenylation signal (AATAAA) preceding the polyA$^+$. This indicates that the 3' end of the cDNA is intact.

At the 5' end, the translation start site was determined on the basis that an in-frame stop codon is present about 15 amino acids upstream to the methionine assigned as the +1 site.

The putative protein has 241 amino acids with an estimated molecular weight of 26 kd. It appears to be a soluble protein since the Kyte-Doolittle plot did not reveal any hydrophobic segments which can serve as transmembrane domains. A BLAST search of the NCBI, EMBL and SWISSPORT databases did not find significant homology with any known proteins. A MOTIF search identified two potential glycosylation sites (position 18 and 103) and a potential PKC phosphorylation site (position 144).

FIG. 12 shows a restriction map of inaF cDNA and of the corresponding genomic region in the A23 clone and three inaF mutants.

EXAMPLE 10

Immunodetection of the TRP protein

To determine if inaF mutations affect the amount of the TRP protein, Western blot analyses were performed. The blot was probed with a monoclonal anti-TRP antibody described in Pollock, *J. Neurosci.* 15:3747-3760 (1995). Results showed that the TRP protein is reduced to about 15% and 10% of the wild type level in *inaF*^{P105p} and *inaF*^{P106x}, respectively, at 1 day post-eclosion (FIG. 13). The reductions are not due to non-specific reductions of retinal proteins. Other retinal proteins examined [rhodopsin, PLCB

(NORPA), and InaD) did not show similar reductions at this age (data not shown), nor were there any signs of retinal degradation in such young flies.

EXAMPLE 11

Recombinant Expression Vectors Encoding InaF

A glutathione-S-transferase-InaF polypeptide (GST-InaF) fusion construct was made by ligating the *inaF* coding region in frame with the glutathione transferase gene in the pGEX-KG vector [(Guan and Dixon, *Anal. Biochem.*, 192:262-267 (1991)]. Following transformation of bacteria (E. coli BL-21), over expression of the fusion protein was achieved by induction with IPTG. The fusion protein was partially purified by using immobilized glutathione [Guan and Dixon (1991), cited above]. Further purification can be achieved by ion exchange chromatography. In order to obtain purified InaF protein, the fusion protein can be digested with thrombin (Sigma) and the InaF protein can be eluted from an immobilized glutathione agarose column as known in the art.

Biological Deposit Under The Budapest Treaty

A deposit of inaF cDNA, designated as inaF cDNA-1/XL-1 Blue was deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209. The deposit is Epicurian Coli XL-1 Blue (Stratagene) harboring inaF cDNA (SEQ ID:1, nucleotides 314 to 1036) from Drosophila melanogaster (Berlin) in a

pBluescript II KS (Stratagene) vector. The accession number is ATCC 207232.

While the invention has been illustrated and described in detail in the drawings and foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiment has been shown and described and that all changes and modifications that come within the spirit of the invention are desired to be protected. In addition, all references cited herein are indicative of the level of skill in the art and are hereby incorporated by reference in their entirety.

CLAIMS

What is claimed is:

1. An isolated nucleic acid molecule, comprising a nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells, said nucleotide sequence having substantial similarity to the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

2. The molecule of claim 1, wherein said nucleotide sequence is comprised of the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

3. The molecule of claim 1, wherein said protein is comprised of an amino acid sequence having at least about 30% identity with the amino acid sequence set forth in SEQ ID:1.

4. The molecule of claim 1, wherein said protein is comprised of an amino acid sequence having at least about 50% identity with the amino acid sequence set forth in SEQ ID:1.

5. The molecule of claim 1, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1.

6. The molecule of claim 1, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1 or a sufficiently similar sequence thereto to exhibit the ability to regulate calcium ion entry into cells.

7. An isolated nucleic acid molecule, comprising a nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells, said nucleotide sequence having the sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

8. An isolated nucleic acid molecule, comprising a nucleotide sequence having at least about 80% identity to a 400 nucleotide long sequence within the sequence set forth in SEQ ID:1 from nucleotide 301 to nucleotide 1036, said nucleotide sequence from nucleotide 301 to nucleotide 1036 encoding a protein functioning in regulating calcium entry into cells.

9. A recombinant nucleic acid molecule, comprising a nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells, said nucleotide sequence having substantial similarity to the sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

10. The molecule of claim 9, wherein said nucleotide sequence is comprised of the sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

11. The molecule of claim 9, wherein said protein is comprised of an amino acid sequence having at least about 30% identity with the amino acid sequence set forth in SEQ ID:1.

12. The molecule of claim 9, wherein said protein is comprised of an amino acid sequence having at least about 50% identity with the amino acid sequence set forth in SEQ ID:1.

13. The molecule of claim 9, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1.

14. The molecule of claim 9, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1 or a sufficiently similar sequence thereto to exhibit the ability to regulate calcium ion entry into cells.

15. The molecule of claim 9, further comprising a promoter operably linked to a terminal 5' end of said nucleotide sequence.

16. The molecule of claim 15, wherein said promoter is selected from the group consisting of a constitutive promoter, an inducible promoter, and a cell-specific promoter.

17. A recombinant nucleic acid molecule, comprising a nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells, said nucleotide sequence having the sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

18. A host cell, comprising an introduced nucleic acid molecule having a nucleotide sequence of substantial similarity to the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036, said nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells.

19. The host cell of claim 18, wherein said nucleotide sequence is comprised of the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

20. The host cell of claim 18, wherein said protein is comprised of an amino acid sequence having at least about 30% identity with the amino acid sequence set forth in SEQ ID:1.

21. The host cell of claim 18, wherein said protein is comprised of an amino acid sequence having at least about 50% identity with the amino acid sequence set forth in SEQ ID:1.

22. The host cell of claim 18, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1.

23. The host cell of claim 18, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1 or a sufficiently similar sequence thereto to exhibit the ability to regulate calcium ion entry into cells.

24. A host cell, comprising an introduced nucleic acid molecule having a nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036, said nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells.

25. A purified InaF protein.

26. A purified protein, said protein having an amino acid sequence having at least about 30% identity to the amino acid sequence set forth in SEQ ID:1, said protein functioning in regulating calcium ion entry into cells.

27. The protein of claim 26, wherein said protein has an amino acid sequence as set forth in SEQ ID:1.

28. The protein of claim 26, wherein said protein has an amino acid sequence having at least about 50% identity to the amino acid sequence set forth in SEQ ID:1.

29. The protein of claim 26, wherein said protein has an amino acid sequence as set forth in SEQ ID:1 or a sufficiently similar amino acid sequence thereto to exhibit the ability to regulate calcium ion entry into cells.

30. A purified protein, said protein having an amino acid sequence set forth in SEQ ID:1, said protein functioning in regulating calcium ion influx into cells.

31. A purified protein, said protein having an amino acid sequence encoded by a nucleic acid molecule having a nucleotide sequence of substantial similarity to the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036, said protein functioning in regulating calcium ion entry into cells.

32. A recombinant protein, comprising:
an amino acid sequence having at least about 30% identity to the amino acid sequence set forth in SEQ ID:1, said protein functioning in regulating calcium ion entry into cells.

33. The protein of claim 32, wherein said protein has an amino acid sequence having at least about 50% identity to the amino acid sequence set forth in SEQ ID:1.

34. A method of expressing an InaF protein, said method comprising:

(a) introducing into a host cell a nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells, said nucleotide sequence having substantial similarity to the sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036; and

(b) culturing under conditions to achieve expression of said protein.

35. The method of claim 34, wherein said nucleotide sequence is comprised of the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

36. The method of claim 34, wherein said protein is comprised of an amino acid sequence having at least about 30% identity with the amino acid sequence set forth in SEQ ID:1.

37. The method of claim 34, wherein said protein is comprised of an amino acid sequence having at least about 50% identity with the amino acid sequence set forth in SEQ ID:1.

38. The method of claim 34, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1.

39. The method of claim 34, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1 or a sufficiently similar sequence thereto to exhibit the ability to regulate calcium entry into cells.

40. The method of claim 34, wherein said nucleotide sequence is inserted in a vector.

41. The method of claim 40, wherein said vector is a plasmid vector.

42. A method of expressing an InaF protein, said method comprising:

(a) introducing into a host cell a recombinant nucleic acid molecule comprising a nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells, said nucleotide sequence having substantial similarity to the sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036; and

(b) culturing under conditions to achieve expression of said protein.

43. The method of claim 42, wherein said nucleotide sequence is comprised of the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

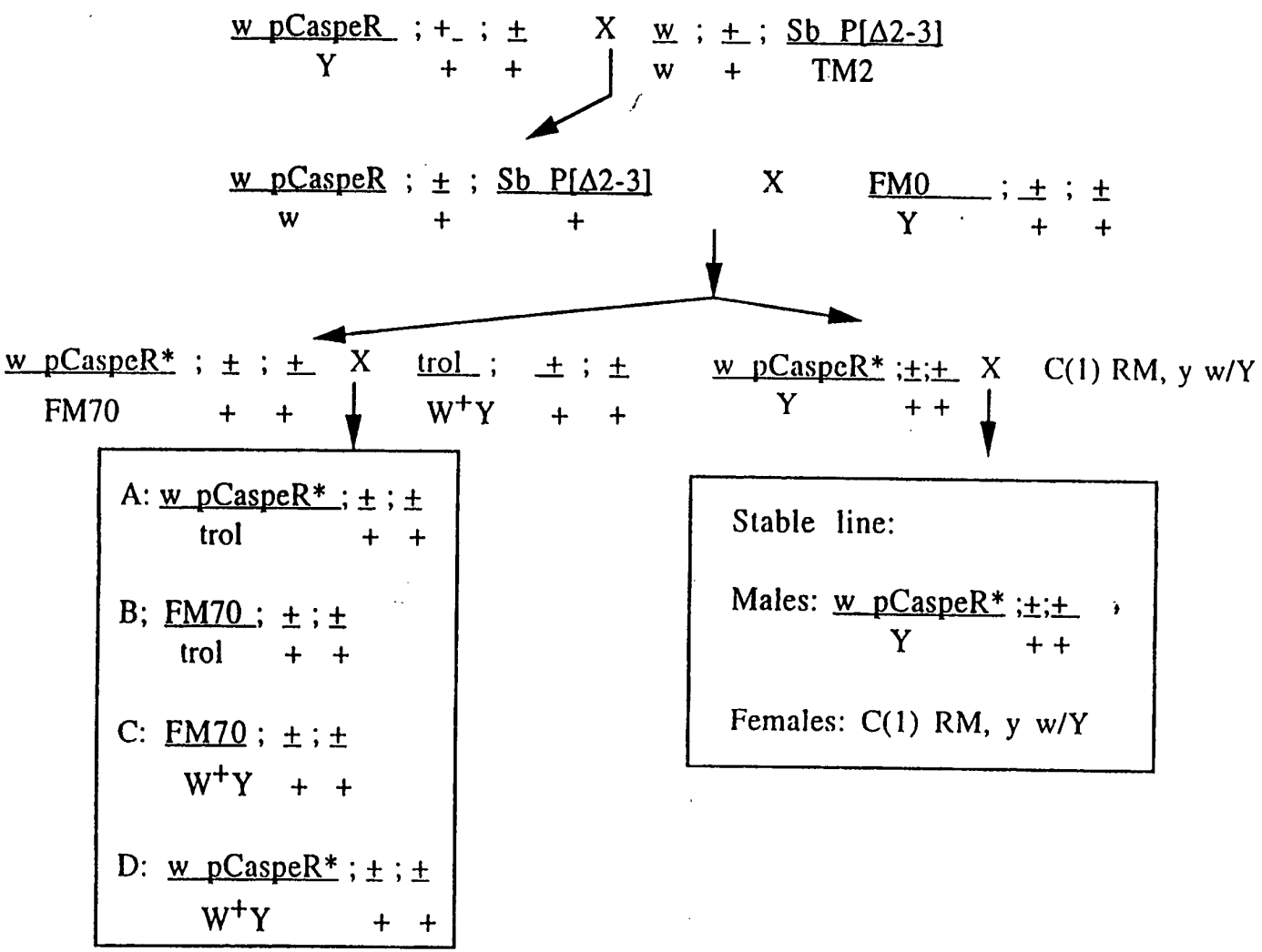


Fig. 1

PCT/US99/10821

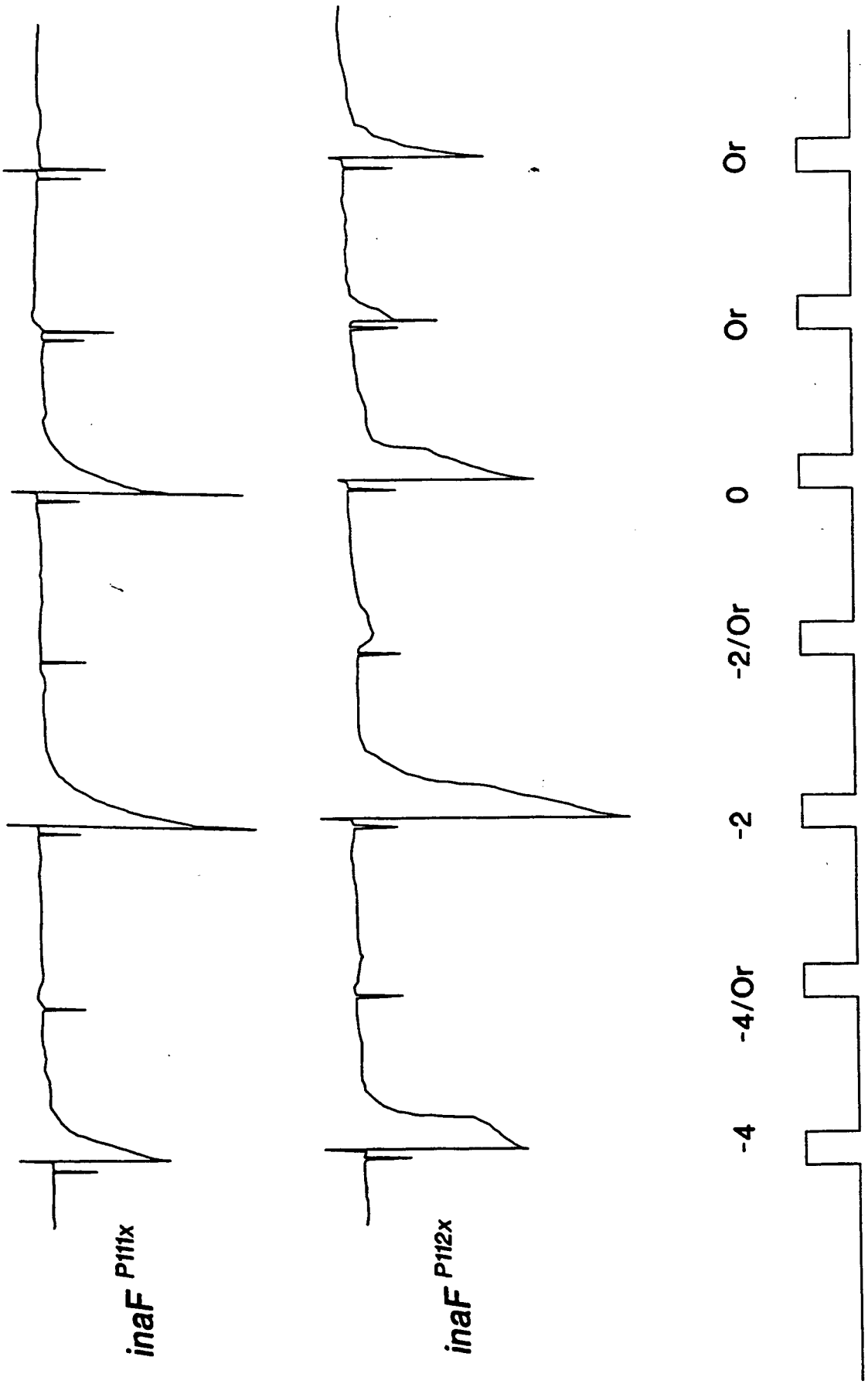


Fig. 2

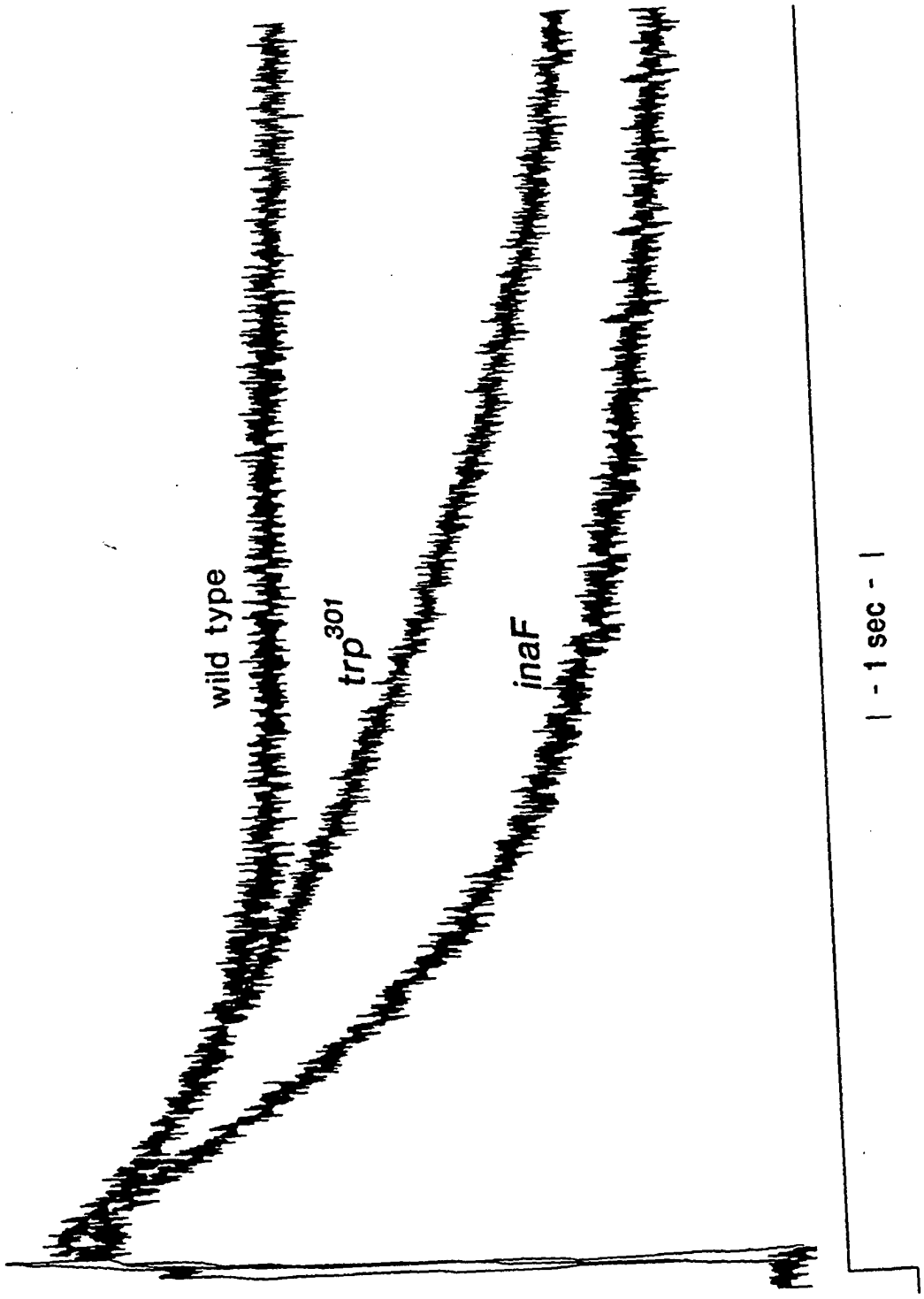


Fig. 3

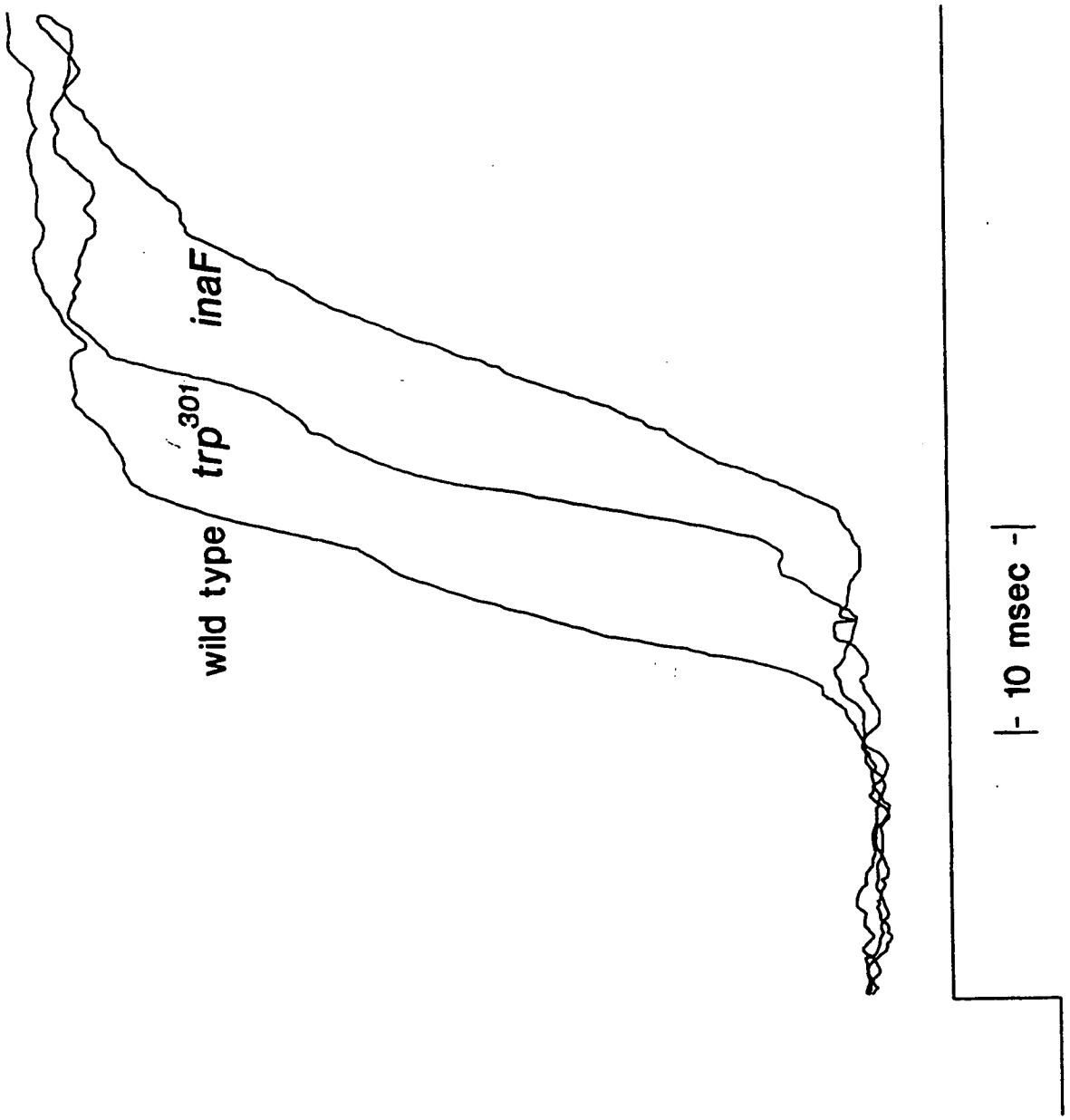


Fig. 4

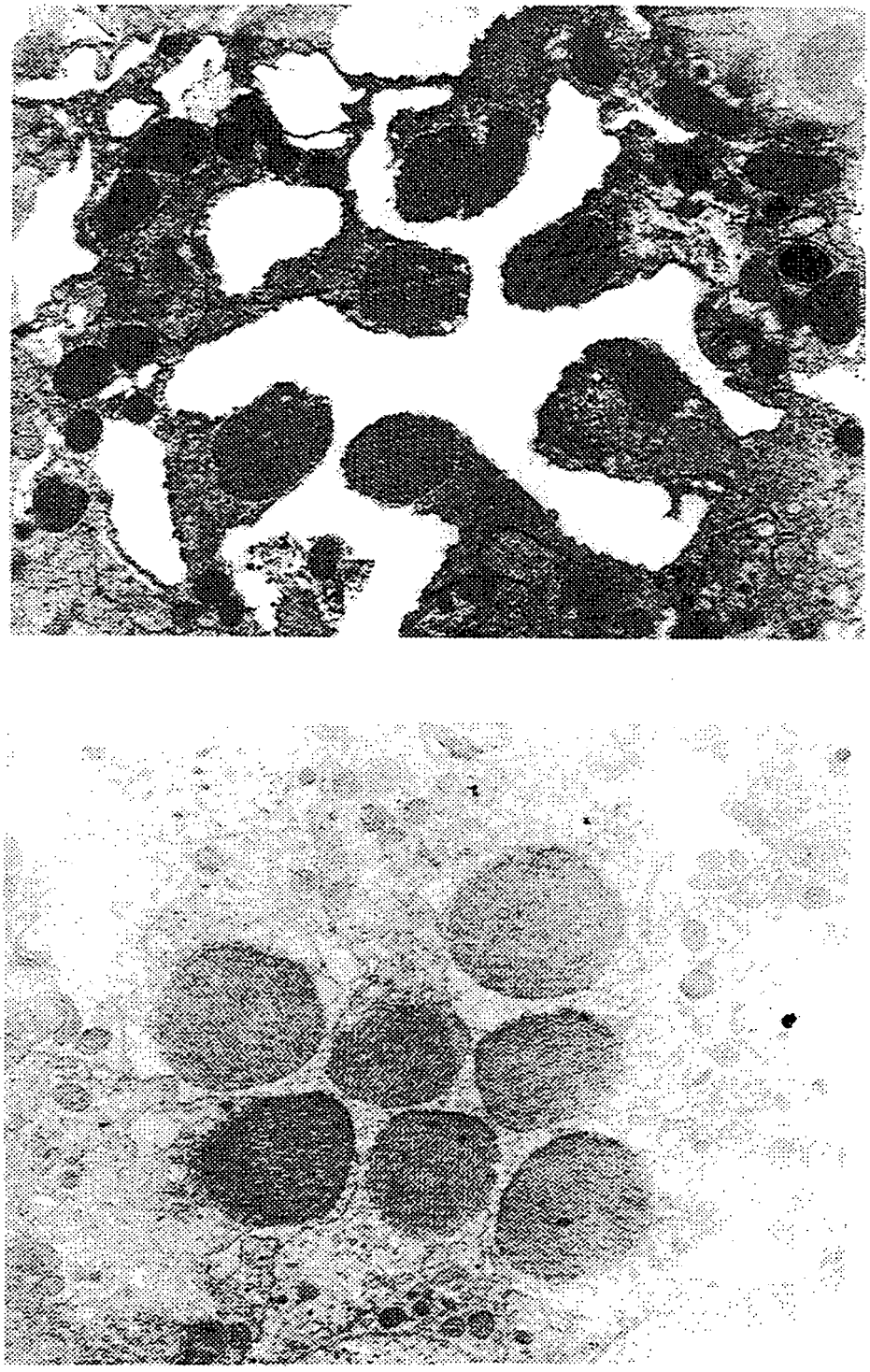
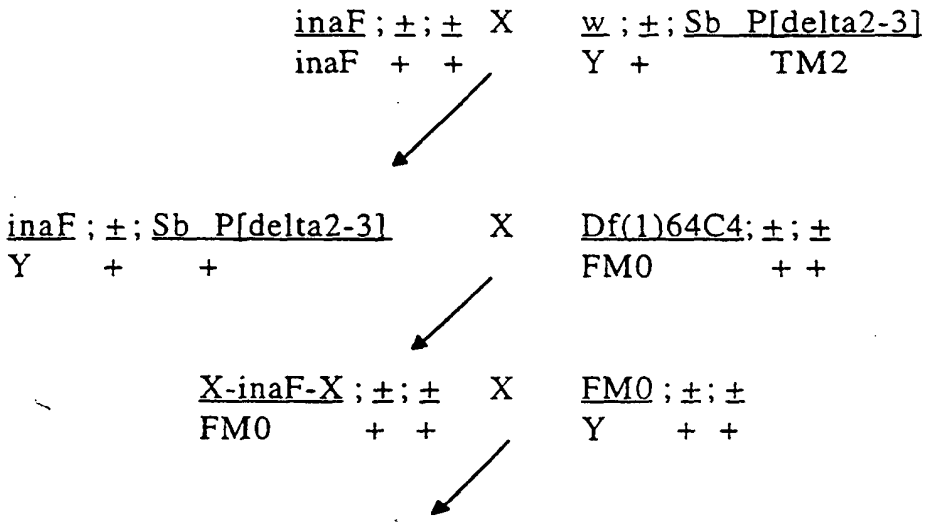


Fig. 5A

inaF;bw;st 19 days old
Light/Dark reared

Fig. 5B



A: X-inaF-X ; ± ; ±
FM0 + +

B: FM0 ; ± ; ±
FM0 + +

C FM0 ; ± ; ±
Y + +

D X-inaF-X ; ± ; ±
Y + +

Fig. 6

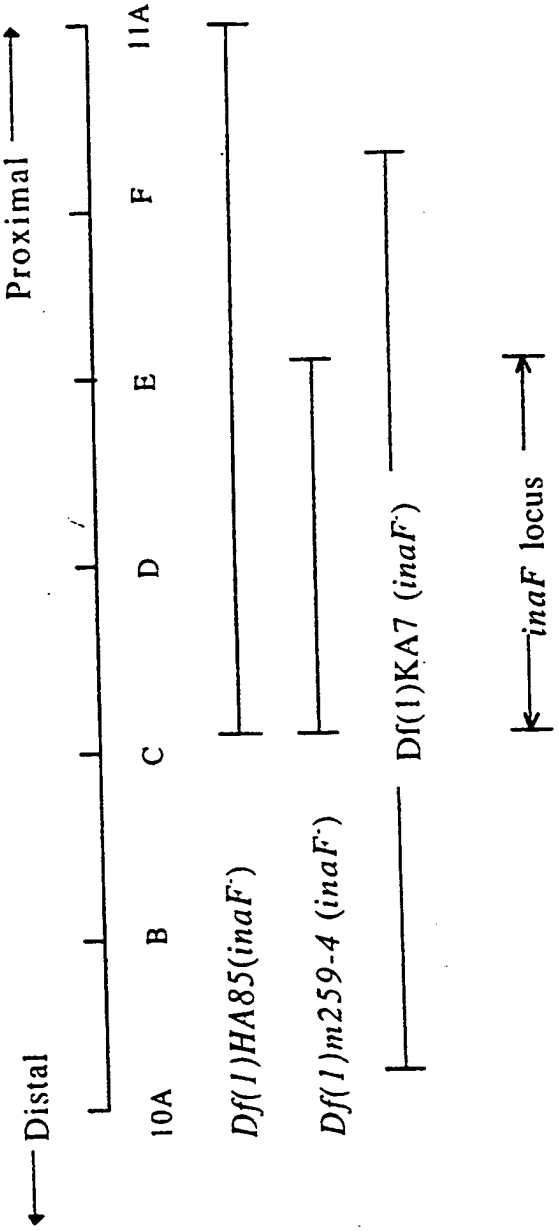

Proximal
Distal
10A
B
C
D
E
F
11A
Df(1)HA85(inaF-)
Df(1)m259-4 (inaF-)
Df(1)KA7 (inaF-)
inaF locus

Fig. 7

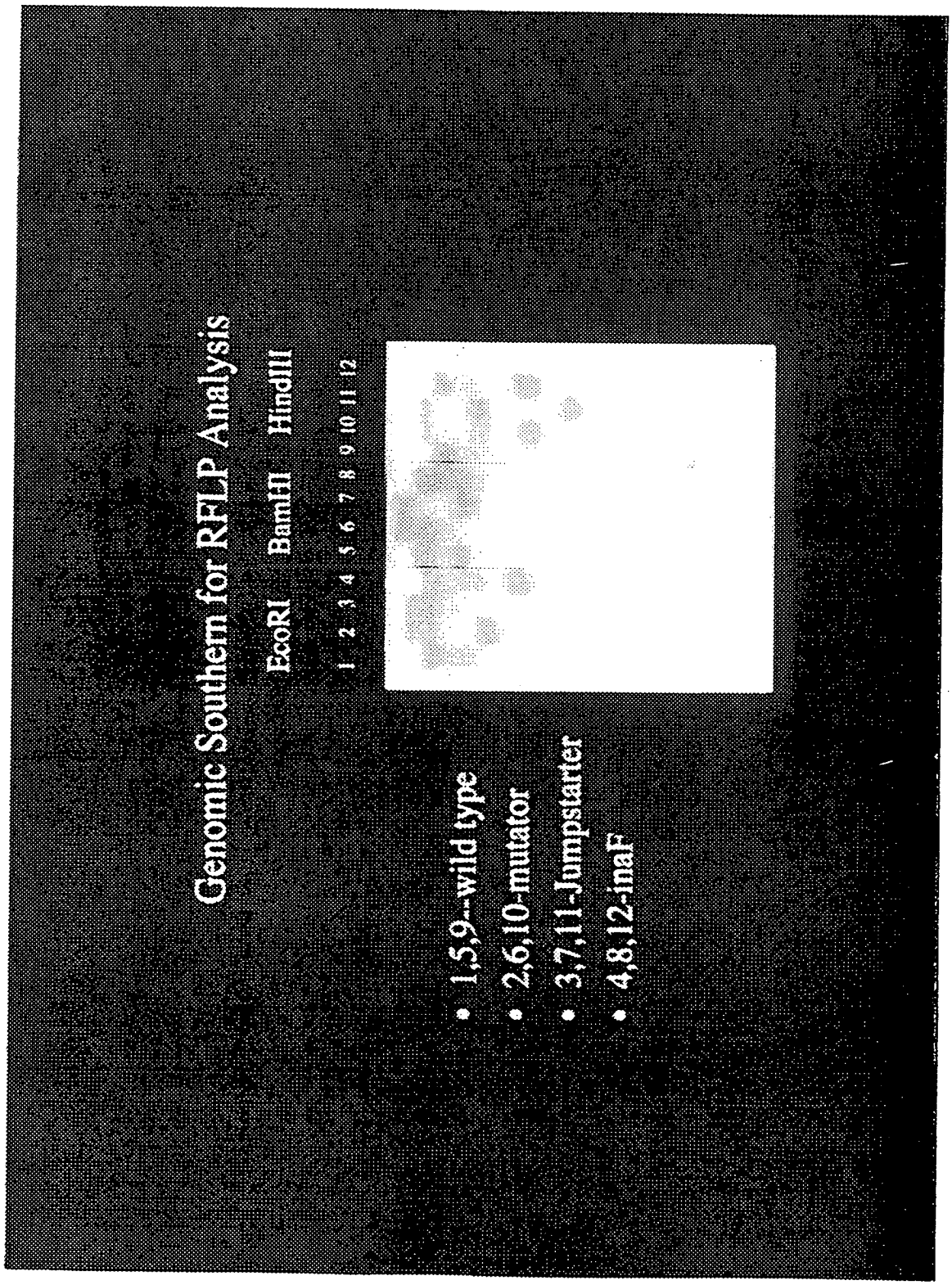
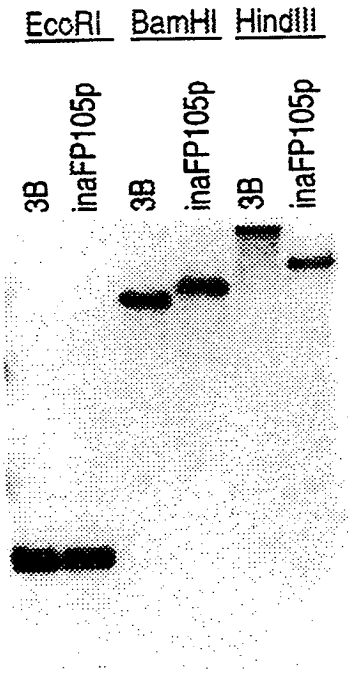

Genomic Southern for RFLP Analysis
EcoRI BamHI HindIII
1 2 3 4 5 6 7 8 9 10 11 12
• 1,5,9--wild type
• 2,6,10-mutator
• 3,7,11-Jumpstarter
• 4,8,12-inaF

Fig. 8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HindIII
3B
inaFP105p
3B
inaFP105p
3B
inaFP105p

Fig. 9

Fig. 10

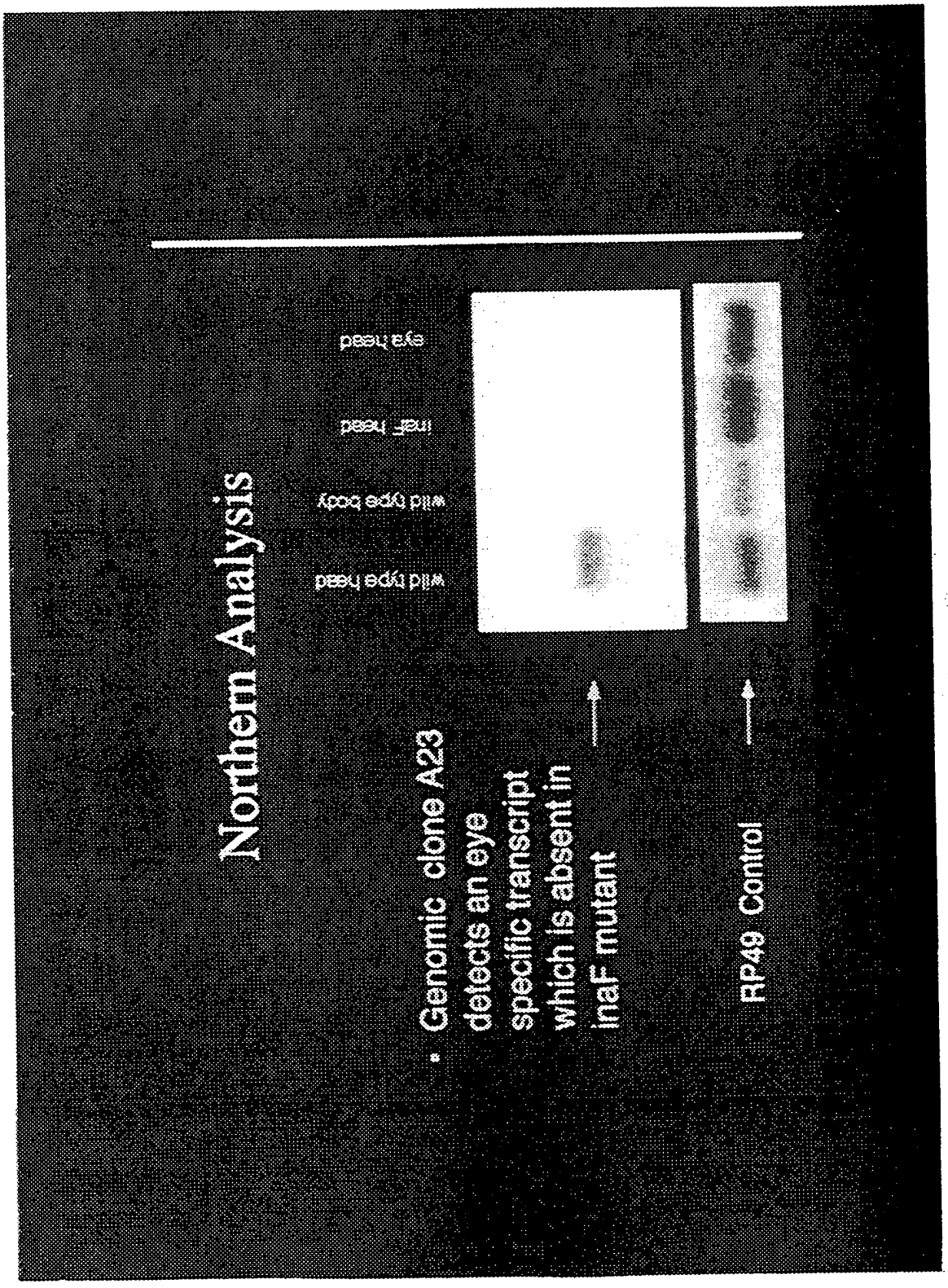
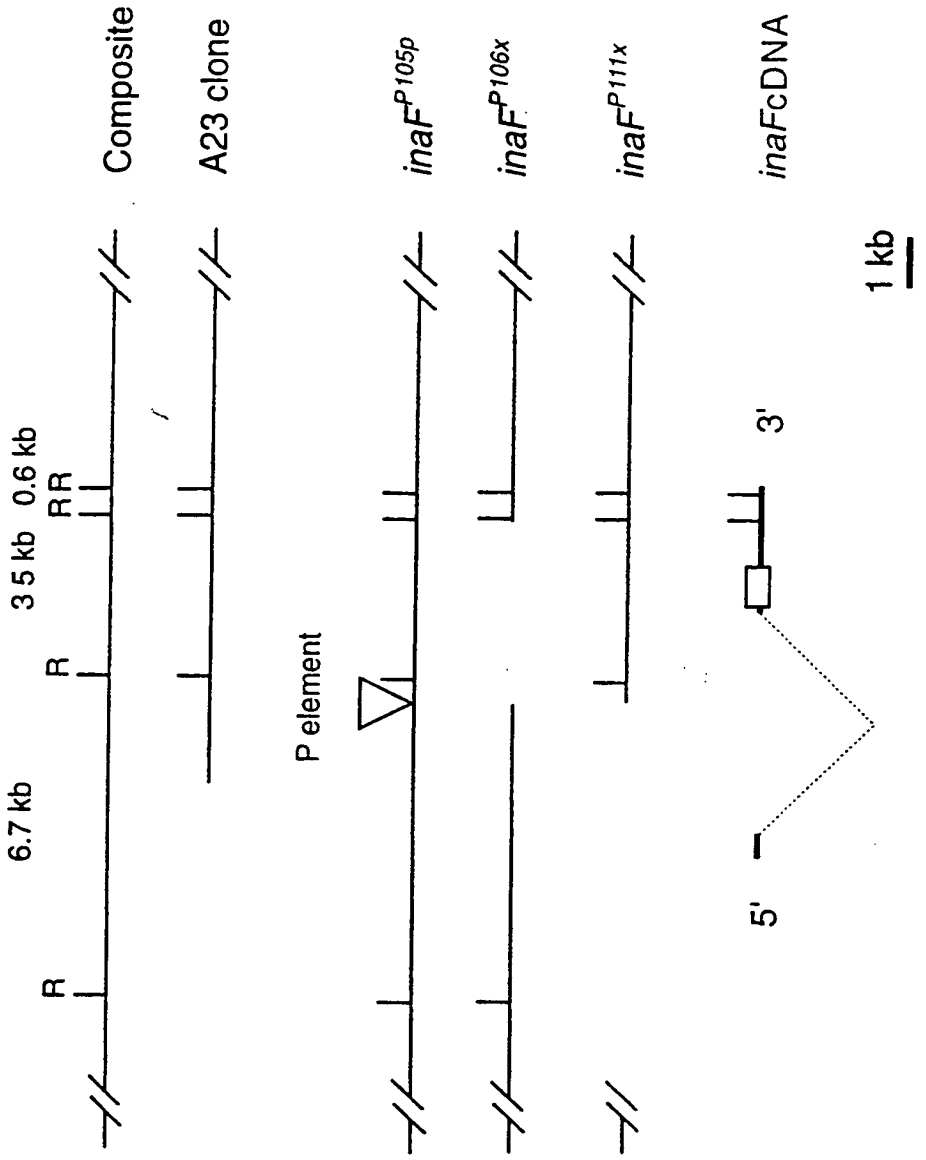

Northern Analysis
Genomic clone A23 detects an eye specific transcript which is absent in inaF mutant
wild type head
wild type body
inaF head
eye head
RP49 Control

Fig. 11


Composite
A23 clone
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inaF^P106x
inaF^P111x
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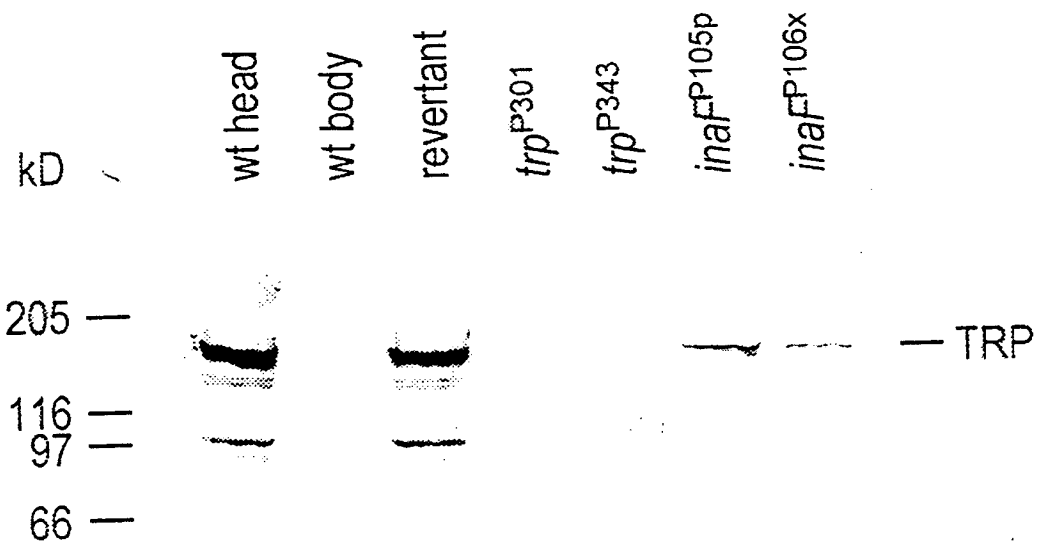
3.5 kb
0.6 kb
R
R
R
P element
5'
3'
1 kb

Fig. 12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wt head
wt body
revertant
trp^P301
trp^P343
inaF^P105p
inaF^P106x
205
116
97
66
TRP

Fig. 13

SEQUENCE LISTING

<110> Pak, William L.
Li, Chenjian
Geng, Chaoxian

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<130> 7024-384/PUR-92

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1 5 10

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Leu Gln Leu Gln Gln Leu Glu Ala Asn Asn Arg Phe Gln Glu Val Phe
15 20 25

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Ala Thr Ala Thr Ile Ile Gln Ala His Pro His Pro His Pro His Pro
30                  35                  40                  45

agg gag ccg ccc aag aag ccg ctt tta gga cca tat agc ccg caa ccc        496
Arg Glu Pro Pro Lys Lys Pro Leu Leu Gly Pro Tyr Ser Pro Gln Pro
                50                  55                  60

ggc aac ata agt cac gct atg ggt ggt gat cag ttg gat gca gaa acg        544
Gly Asn Ile Ser His Ala Met Gly Gly Asp Gln Leu Asp Ala Glu Thr
            65                  70                  75

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Glu Gln Gly His Met Pro Leu Ile Leu Asp Thr Ser Pro Pro Val Glu
        80                  85                  90

gta acc gga atg ggt cac ctg aag cgg aag aca cat cgc ggt cac tac        640
Val Thr Gly Met Gly His Leu Lys Arg Lys Thr His Arg Gly His Tyr
    95                  100                 105

aaa cat cat aga gcc cga gcc ggt ggt caa aag aaa ctg tcc att gcc        688
Lys His His Arg Ala Arg Ala Gly Gly Gln Lys Lys Leu Ser Ile Ala
110                 115                 120                 125

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Asn Ser Met Ala Ser Ser Thr Pro Ser Thr Thr Ala Gly Gly Asp Ala
                130                 135                 140

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Ser Met Ala Thr Ala Ala Thr Leu Pro His Gly Tyr Met Asp Ala Pro
            145                 150                 155

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Leu Asn Pro Ala Ala Gly Thr Ile Val Gln Ala Pro Gln Leu Gln Leu
        160                 165                 170

tac acc tcg atg ccc att cca ctg atc ctg agt ccc agc gac gaa aag        880
Tyr Thr Ser Met Pro Ile Pro Leu Ile Leu Ser Pro Ser Asp Glu Lys
    175                 180                 185

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Arg Pro Ser His His Ala His Gly His Val His Gly Glu Arg Arg Asn
190                 195                 200                 205

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Gly Tyr Glu Ala Gln Thr Tyr Leu Asn Pro Phe Leu Thr Gly Glu Leu
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Ile Phe Glu Lys
240

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tataatctca gttaatgcgt agaatgcgtt tgcaattgaa caaatttaac gttttatagc 1436

aagaacttag gaacaaaagt tgtaatcgct tattctataa taatgttaac taaagccaaa 1496

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Pro Lys Lys Pro Leu Leu Gly Pro Tyr Ser Pro Gln Pro Gly Asn Ile
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His Met Pro Leu Ile Leu Asp Thr Ser Pro Pro Val Glu Val Thr Gly
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Met Gly His Leu Lys Arg Lys Thr His Arg Gly His Tyr Lys His His
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Met Pro Ile Pro Leu Ile Leu Ser Pro Ser Asp Glu Lys Arg Pro Ser
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